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(54) Title: METHODS FOR TREATING AND/OR PREVENTING ALZHEIMER'S DISEASE USING PHENOTHIAZINES AND/OR THIOXANTHENES

(57) Abstract

Disclosed are methods for preventing or treating Alzheimer's Disease comprising administering to a patient an amount of a phenothiazine or a thioxanthene effective to prevent or diminish the accumulation of abnormally phosphorylated, paired helical filament epitopes associated with Alzheimer's Disease.

METHODS FOR TREATING AND/OR PREVENTING ALZHEIMER'S DISEASE USING PHENOTHIAZINES AND/OR THIOXANTHENES

This is a continuation-in-part application of U.S. Application Serial No. 08/287,339, filed August 8, 1994, which is a continuation-in-part of U.S. Application Serial No. 08/042,425, filed March 26, 1993, the contents of both of which are incorporated by reference herein.

Statement of Government Interest

This invention was made with government support under Grant Nos. NIH AG 06803 and NIH MH 38623. As such, the government has certain rights in this invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to methods for preventing or treating Alzheimer's Disease. More specifically, the methods comprise administering to a patient an amount of a phenothiazine or a thioxanthene effective to prevent or diminish the accumulation of abnormally phosphorylated, paired helical filament epitopes associated with Alzheimer's Disease.

2. Description of the Related Art

Alzheimer's Disease is a progressive neurodegenerative disorder affecting 7% of the population over 65 years of age and characterized clinically by progressive loss of intellectual function. Alzheimer's Disease is characterized by the accumulation of abnormally phosphorylated, paired helical filaments (PHFs), which accumulate as neurofibrillary tangles in neuronal cell bodies and in the neurites of the neuritic plaques. PHFs also are present in neuronal processes, such as axons and dendrites. As much as 90% of the PHF in the cortex of the average Alzheimer case is present in the neuronal processes rather than in the plaque or tangle (Wolozin, B.L. and Davies, P. Ann. Neurol. 22:521-526 (1987)).

It has been suggested that the accumulation of PHFs indicative of Alzheimer's Disease begins in the entorhinal cortet years before the clinical signs of the disease are

apparent (Braak, H., et al. Neurosci Lett. 103:24-28 (1989); Braak, H., et al. Neuropath. and Applied Neurobiol. 15:13-26 (1989); Braak, H., et al. Acta Neuropath. 82:239-259 (1991)). In the initial stages of Alzheimer's Disease, only those neurons of the entorhinal cortex contain PHF. In the next stages, when the patient shows some short term memory loss, the neurons of the CA1 and CA2 pyramidal layer of the hippocampus contain these structures. At later stages, neurons of the association cortex begin to show evidence of PHF formation. The prevention of the progression of Alzheimer's Disease at the early stages, or when the PHF pathology is largely limited to the hippocampus by preventing the accumulation of PHFs, would be a major benefit to the Alzheimer's patient.

Early attempts to determine the molecular nature of PHFs from isolated neurofibrillary tangle were hindered by difficulties in PHF isolation and solubility due to the complexity of the tangle (Yen, S-H, et al. NY Acad. Sci. 455:819-825 (1985); Crowther, T., et al. Ann. Med. 21:127-132 (1989); Vogelsang, G.D., et al. Clin. Biol. Res. 317:791-800 (1989)). Despite these difficulties sequences homologous to the microtubule-associated protein, tau, have been obtained indicating that PHFs contain this protein (Crowther, T., et al. Ann. Med. 21:127-132 (1989)). Others have shown that the mature neurofibrillary tangle contains at least two other proteins in addition to tau (Vogelsang, G.D., et al. Prog. Clin. Biol. Res. 317:791-800 (1989); Iqbal, K. and Grundke-Iqbal, I. Molecular Neurobiology 5:399-410 (1991); Sternberger, L.A., et al. Prog. Clin. Biol. Res. 317:763-768 (1989); Bancher, C., et al. Prog. Clin. Biol. Res. 317:913-924 (1989)).

The involvement of abnormal protein phosphorylation in the formation of PHF in Alzheimer's patients has been suggested by a variety of studies which have shown that tau in PHF is hyperphosphorylated (Kosik, K.S., et al. Ann. Med. 21:109-112 (1989); Kosik, K.S. J. Gerontol. 44:55-58 (1989); Goedert, M. Trends in Neurosciences 16:460-464 (1993); Iqbal,

K. Act Neurobiologiae Exp. 53:325-335 (1993)). There also is evidence that other proteins, especially neurofilaments and other microtubule associated proteins (MAPs), also are hyperphosphorylated in the brains of patients with Alzheimer's Disease (Grundke-Iqbal, I. and Iqbal, K. Prog. Clin. Biol. Res. 317:745-753 (1989); Onorato, M., et al. Prog. Clin. Bio. Res. 317:781-789 (1989); Seitelberger, F., et al. J. Neural Transmission 33:37-33 (1991); Lovestone, S., et al. Curr. Opinions in Neurology and Neurosurgery 5:833-888 (1992)), suggesting that the formation of PHFs results from the abnormal phosphorylation of these proteins, as well.

Monoclonal antibodies have been generated by the inventors of the present invention which are reactive with PHFs in the brain tissue of Alzheimer's Disease patients, and in most cases, minimally or not cross reactive with proteins in the normal adult brain. These antibodies react with abnormally phosphorylated epitopes on PHF proteins in the Alzheimer's brain which are modified as a result of the disease process.

The ability to inhibit the production of abnormally phosphorylated PHF proteins including tau, other MAPs and neurofilament proteins by blocking epitopes recognized by these antibodies is expected to interfere or prevent the formation and acccumulation of PHF, and therefore inhibit or prevent the progression of Alzheimer's disease. To date, however, it has not been possible to consistently induce the formation of abnormally phosphorylated PHFs epitopes in abundance either *in vivo* or *in vitro*.

Shea, T.B., et al. reported that aluminum salts induce the accumulation of neurofilaments in neuroblastoma cells (Shea, T.B., et al. Brain Res. 492:53-64 (1989)). The neurofilaments produced by Shea, et al., however, have not been shown to correlate with PHFs associated with Alzheimer's Disease.

Ko, Li-wen, et al. reported the alleged expression of epitopes associated with neurons in Alzheimer's Disease in the human neuroblastoma cell line IMR32 grown in a

differentiation medium using polyclonal antibodies to PHF epitopes (Ko, Li-wen, et al. Am. J. Path. 136(4):867-879 (1990)). The reactivity of the IMR32 cultured cells with Alz-50, however, was equivalent in the control media as well as in other cell lines that Ko, et al. reported did not express PHF epitopes, indicating that the level of expression was too low to represent a model for Alzheimer's Disease.

Hence, a great need has arisen for a model system which consistently and abundantly induces the formation of abnormally phosphorylated PHFs epitopes, which can be used to screen for compounds which block the production of these epitopes. The present invention, which is directed to a tissue culture model in which Alzheimer's Disease epitopes are continually and abundantly expressed, fulfills a great need in this field.

SUMMARY OF THE INVENTION

The present invention provides an *in vitro* model cell system which expresses abnormally phosphorylated, paired helical filament epitopes associated with Alzheimer's Disease, which comprises neuroblastoma cells which have been treated with an amount of a protein phosphatase inhibitor effective to render homogenate from the cells treated therewith immunoreactive with antibodies which specifically bind to abnormally phosphorylated, paired helical filament epitopes. The model system of this invention may be used for determining whether an agent is capable of preventing or diminishing the accumulation of abnormally phosphorylated, paired helical filament epitopes associated with Alzheimer's Disease. The present invention also provides a method for treating or preventing Alzheimer's Disease which comprises the administration of a drug which blocks the accumulation of abnormally phosphorylated, paired helical epitopes associated with Alzheimer's Disease.

BRIEF DESCRIPTION OF THE FIGURES

The above description, as well as further objects and features of the present invention, will be more fully understood by reference to the following detailed description of the presently preferred, albeit illustrative, embodiments of the present invention when taken in conjunction with the accompanying drawings wherein:

Figure 1 represents the time course for OkA treatment. Confluent layers of MSN cells were incubated at 37°C for 0 minutes and 4 hours with the KRP containing 0.05% DMSO (Controls), or 30 minutes, 90 minutes, 4 hours, 10 hours, and 24 hours with 1 μM OkA. The cells were harvested and boiled to generate heat-stable fractions. Twenty μg of protein per lane was resolved by SDS-PAGE, and gels were electrophoretically transferred to nitrocellulose. Blots were immunostained with the Alz-50, Tau-1, 188, PHF-1, NP-8, and T3-P antibodies. The arrow beside the PHF-1 blot points to the 68-kDa protein, which is only apparent after OkA treatment.

Figure 2 represents the dose-response curve for OkA treatment. MSN cells were incubated with 5 nM, 50 nM, 1 μM, and 50 μM OkA in KRP for 4 hours at 37°C. Heat-stable fractions were prepared and used for western blot analysis as in Figure 1. Note that the 50 μM OkA-treated sample in the Alz-50 blot represents only 13 μg of protein.

Figure 3 represents the effects of protein synthesis inhibition. MSN cells were incubated at 37°C for 90 minutes with 1 μM OkA in the absence or presence of 100 μM anisomycin. Heat-stable fractions were prepared, and 30 μg of protein was analyzed by western blotting as in Figure 1.

Figure 4 represents the treatment of MSN cell lysates with OkA: Effects of alkaline phosphatase. MSN cells were collected by centrifugation and resuspended in KRP containing 4 mM PMSF, 25 μM leupeptin, and 2 mM EGTA. The cell suspension was sonicated for 2 seconds, and the resulting lysate was incubated at 37°C for 60 minutes in the absence or

presence of 10 μM OkA. Additional samples were treated with 50 nM OkA. Thirty μg of the respective heat-stable fractions were loaded per lane. Duplicate blots were incubated at 37°C for 24 hours with 20 units of alkaline phosphatase per ml of pH 8.0 buffer in the presence of 10 mM PMSF (+). The blots were then immunized with the Alz-50, Tau-1, 188, PHF-1, T3P, and NP-8 antibodies.

Figure 5A represents the ^{32}P-labeling of tau using pulse-chase. MSN cells were preincubated at 37°C for one hour with 2.5 mCi of ^{32}P-orthophosphoric acid in phosphate-free medium. The cells were then chased for 90 minutes with KRP in the absence (control) or presence of 1 μM OkA. Heat-stable supernatants were further enriched for tau by perchloric acid (PCA) treatment and methanol precipitation as described in the Experimental Details Section. Blots were stained with Alz-50 and then subjected to autoradiography.

Figure 5B represents the ^{32}P-labeling of tau using pulse. Cells were preincubated for 30 minutes with labeled orthophosphoric acid at the end of which time, 1 μM OkA acid was introduced into one sample. Following a further 90 minute incubation, heat-stable supernatants were isolated and subjected to PCA and methanol precipitation. Blots were stained with Alz-50 and subsequently subjected to autoradiography.

Figure 6 represents the ^{35}S-methionine labelling of tau. MSN cells were pulse labeled overnight in methionine-free medium containing 200 μCi per ml isotopic methionine. The cells were subsequently chased for 90 minutes with KRP containing 2 mM unlabelled methionine with the indicated additions. Heat-stable supernatants were treated with PCA, and the PCA soluble protein methanol-precipitated for gel-loading. Blots were stained with Alz-50, and subjected to autoradiography.

Figure 7 represents the immunoreactivity of antibodies Alz-50, PHF-1, TG3, TG4, MC2, MC6, and MC15 with normal and Alzheimer's Disease brain, and with MSN cells before (MSN-) and after treatment (MSN+) with okadaic acid.

The data from brain tissue are the mean values derived from studies of mid-temporal cortex from five normal cases and five cases of Alzheimer's Disease. Values from MSN cells are from a single experimental series in which 1 micromolar okadaic acid was added to triplicate MSN cell cultures for 90 minutes prior to harvest of the cells. Each sample (tissue or cell homogenate) was assayed at 8 dilutions, in duplicate. The units on the Y axis are arbitrary units of immunoreactivity.

Figure 8 represents the immunoblot of neuroblastoma (MSN-A) cells expressing paired helical filament epitopes as a control, and after treatment with trifluoperazine and chlorpromazine.

Figure 9A represents relative ADAP concentrations in Broadman area 10 in Alzheimer's Disease patients (AD), normal controls (NC), and schizophrenic patients on chlorpromazine (RX).

Figure 9B represents relative ADAP concentrations in Broadman area 38 in Alzheimer's Disease patients (AD), normal controls (NC), and schizophrenic patients on chlorpromazine (RX).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an *in vitro* model cell system which expresses abnormally phosphorylated, paired helical filament epitopes associated with Alzheimer's Disease, said system comprising neuroblastoma cells which have been treated with an amount of a protein phosphatase inhibitor effective to render homogenate from the cells treated therewith immunoreactive with antibodies which specifically bind to abnormally phosphorylated, paired helical filament epitopes.

The model system of the present invention is produced by treating neuroblastoma cells with an amount of a protein phosphatase inhibitor effective to render homogenate from the cells treated therewith immunoreactive with antibodies which specifically bind to abnormally phosphorylated, paired helical filament epitopes.

The neuroblastoma cells useful in the present invention are neuroblastoma cells isolated from the nervous system tumors of children which contain tau, neurofilament proteins, and other neuronal proteins. These cells express express little, if any, abnormally phosphorylated, paired helical filament epitopes until treated with the phosphatase inhibitor. In the particularly preferred embodiment, the neuroblastoma cells are human cells deposited with the American Type Culture Collection under ATTC Accession No. CRL 11253.

The protein phosphatase inhibitor is any inhibitor which induces the expression of abnormally phosphorylated, paired helical filament epitopes associated with Alzheimer's Disease in neuroblastoma cells treated therewith. In the preferred embodiment, the protein phosphatase inhibitor is okadaic acid, calyculin A, microcystin-LR, nodularin and phenylarsine oxide. In the most preferred embodiment, the protein phosphatase inhibitor is okadaic acid.

The amount of protein phosphastase inhibitor used to treat the neuroblastoma cells is an amount effective to induce expression of abnormally phosphorylated, paired helical

filament epitopes in the cells treated with that inhibitor. In the preferred embodiment, the concentration of the inhibitor is between 0.1 and 10 μM. Most preferably, the concentration is 1.0 μM. However, the particular optimal concentration will depend upon the inhibitor and cells used.

The expression of abnormally phosyphorylated, paired helical filament epitopes may be detected using antibodies which specifically bind to abnormally phosphorylated, paired helical filament epitopes. The epitopes are detected by Western blot analyses or by quantitative ELISA as described in the Experimental Details Section. The antibodies may be polyclonal or monoclonal, and are preferably monoclonal. The antibodies may be prepared from PHFs isolated from Alzheimer's patients using known procedures.

In the preferred embodiment, the PHFs are isolated and purified using immunoaffinity as described in Vincent, I.J. and Davies, P. <u>Proc. Natl. Acad. Sci. USA</u> 89:2878-2882 (1992). Particularly, twenty grams of cortical tissue from postmortem Alzheimer's patients is homogenized in 10 vol. of Tris-buffered saline (TBS; 0.1 M Tris base/0.14 M NaCl, pH 7.4) using a Polytron operating at a setting of 5 for two 30 second bursts. The homogeneate is centrifuged at 27,000 x g for 30 minutes at 4°C, and the supernatant is applied to the immunoaffinity column. A monoclonal antibody useful for affinity purification is Ab42 (IgG), a class of switch clone of Alz-50 produced according to published protocol (Spira, G., et al. <u>J. Immunol. Methods</u> 74:307-313 (1984). Approximately 20 mg of protein A-purified Ab42 is dialyzed into 0.1 M sodium phosphate buffer (pH 8.1) and mixed with 10 ml of washed Affi-Gel 10 (Bio-Rad). The mixture is incubated on a rotary shaker for 30 minutes at room temperature. At the end of this time, a volume of 0.1 ethanolamine (pH 8.1) equal to that of the gel/antibody mixture is added and the incubation is continued for 1 hour. The coupling efficiency is generally >90%. The immunoadsorbent is poured into a column and washed with TBS. The column is maintained at 4°C and all chromatography steps are conducted at this temperature. Before application of

sample, the column is treated with at least 2 bed vol. of eluting buffer (3 M potassium thiocyanate) followed by 5 bed vol. of TBS. The 27,000 x g supernatant is loaded onto the immunoaffinity column at a flow rate of approximately 25 ml/hr. Nonspecific binding is reduced by washing the immunoadsorbent with at least 30 bed vol. of TBS. Subsequently, adsorbed protein is eluted with the eluting buffer. Fractions are assayed for protein concentration with the Quantigold protein reagent (Bio-Rad). Peak fractions are dialyzed against TBS and aliquots are stored at -70°C.

To prepare the monoclonal antibodies, mice are immunized with the antigen purified as above by intraperitoneal injection of 10 to 20 micrograms of protein per mouse per injection. Mice are immunized 4 or 5 times before removal of spleens for the production of hybridoma cells by standard protocols. Hybridomas are tested for the production of specific antibodies which react to Alzheimer's brain tissue by ELISA and immunocytochemistry. In the preferred embodiment, the antibodies have high reactivity with Alzheimer's brain tissue and little or no reactivity with brain tissue from normal individuals. In the most preferred embodiment, the antibodies are selected from the group consisting of Alz-50, PHF-1, TG3, TG4, MC2, MC6, and MC15. All of hybridomas secreting these antibodies have been deposited with the American Type Culture Collection, Rockville, Maryland: Alz-50 is secreted from the hybridoma deposited under ATCC Accession Number HB9205; PHF-1 is secreted from the hybridoma deposited under ATCC Accession Number 11743; TG3 is secreted from the hybridoma deposited under ATCC Accession Number 11744; TG4 is secreted from the hybridoma deposited under ATCC Accession Number 11745; MC2 is secreted from the hybridoma deposited under ATCC Accession Number 11737; MC6 is secreted from the hybridoma deposited under ATCC Accession Number 11740; and MC15 is secreted from the hybridoma deposited under ATCC Accession Number 11741.

The model system of the present invention and the antibodies above may be used for determining whether an agent

or drug is capable of preventing or decreasing Alzheimer's Disease activity, namely the accumulation of abnormally phosphorylated, paired helical filament epitopes associated with Alzheimer's Disease.

Accordingly, the present invention also provides a method for determining whether an agent is capable of preventing or diminishing the accumulation of abnormally phosphorylated, paired helical filament epitopes associated with Alzheimer's Disease. That method comprises (a) treating neuroblastoma cells with an amount of a protein phosphatase inhibitor effective to permit expression of abnormally phosphorylated, paired helical filament epitopes in the treated cells, thereby rendering homogeneate from the cells immunoreactive with antibodies which specifically bind to abnormally phosphorylated, paired helical filament epitopes; (b) either concomitantly or thereafter adding to the treated cells an agent suspected of being capable of preventing or diminishing the accumulation of abnormally phosphorylated, paired helical filament epitopes; (c) adding to the treated cells antibodies which specifically bind to abnormally phosphorylated, paired helical filament epitopes associated with Alzheimer's Disease; (d) analyzing homogenate from the cells for immunoreactivity with the antibodies; and (e) determining whether the agent has caused the level of immmunoreactivity between homogenate from the neuroblastoma cells and the antibodies to decrease, said decrease being indicative that the agent has prevented or diminished the accumulation of abnormally phosphorylated, paired helical filament epitopes.

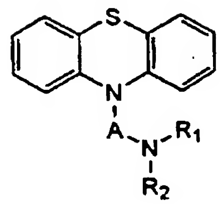
The present invention also provides a screening kit for use in determining whether an agent is capable of preventing or diminishing the accumulation of abnormally phosphorylated, paired helical filament epitopes associated with Alzheimer's Disease, said kit comprising (a) neuroblastoma cells; (b) a protein phosphatase inhibitor which is capable of causing the neuroblastoma cells to express abnormally phosphorylated, paired helical filament epitopes

when the cells are treated therewith, thereby rendering homogenate from the treated cells immunoreactive with antibodies which specifically bind to abnormally phosphorylated, paired helical filament epitopes; and (c) antibodies which specifically bind to abnormally phosphorylated, paired helical filament epitopes expressed by the neuroblastoma cells treated with the protein phosphatase inhibitor.

Using the model system and method of the present invention, it has been found that certain phenothiazine compounds, i.e. trifluoperazine and chlorpromazine (CPZ), cause the level of immmunoreactivity between the homogenate from the neuroblastoma cells and antibodies which specifically bind to abnormally phosphorylated, paired helical filament epitopes expressed by these cells, to decrease. Therefore, since chlorpromazine is effective in decreasing Alzheimer's activity, and the phenothiazines and structurally similar compounds such as thioxanthenes described below display the same profile of activity in the model system and assays described herein as chlorpromazine, the phenothiazines and thioxanthenes are also effective in preventing or decreasing Alzheimer's activity, and therefore are effective in treating or preventing Alzheimer's Disease.

Accordingly, the present invention also provides a method for treating or preventing Alzheimer's Disease in a subject in need of such treatment or prevention which comprises administering an amount of a phenothiazine or a thioxanthene effective to prevent or diminish the accumulation of abnormally phosphorylated, paired helical filament epitopes associated with Alzheimer's Disease.

The phenothiazines useful in the invention have the structural skeleton shown in formula I:

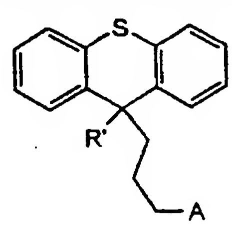


I

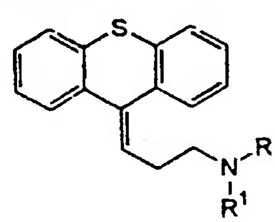
wherein A represents divalent alkylene or alkenylene group having from about 2-8 carbon atoms; and R_1 and R_2 independently represent hydrogen, or organic groups optionally substituted with a variety of moieties such as, for example, halogen, amino, carbonyl, sulfonyl, trifluoromethyl, lower alkyl, lower alkoxyl, etc.; or R_1 and R_2 together with the nitrogen to which they are attached form a five to seven-membered heterocyclic ring, such as, for example, pyrrolidinyl, piperazinyl, lower alkyl piperazinyl, piperidyl, thiomorpholinyl, morpholinyl or hexahydroazepinyl.

In formula I above, the tricyclic ring system may optionally be substituted with a variety of groups such as, for example, halogen, amino, carbonyl, sulfonyl, perfluoroalkyl, such as trifluoromethyl, lower alkyl, and lower alkoxyl, etc. The sulfur atom in formula I above may optionally be present as a sulfoxide.

The thioxanthenes useful in the invention have the structural skeletons shown in formulas II and III:



II



III

wherein R′ is hydrogen or an alkyl group, R and R_1 independently represent hydrogen or alkyl, or R and R_1 together form a 5, 6, or 7-membered heterocyclic ring such as, for example, pyrrolidino, piperidino, morpholino or piperazino; and A is a 5, 6, or 7-membered heterocyclic ring such as, for example, pyrrolidino, piperidino, morpholino or piperazino.

The 5, 6, or 7-membered heterocyclic ring may optionally be substituted with a variety of moieties such as halogen, amino, carbonyl, sulfonyl, perfluoroalkyl, lower alkyl, and lower alkoxy, etc.

In formulas II and III above, the tricyclic ring system may optionally be substituted with a variety of groups such as, for example, halogen, amino, carbonyl, sulfonyl, perfluoroalkyl, such as trifluoromethyl, lower alkyl, and lower alkoxyl, etc.

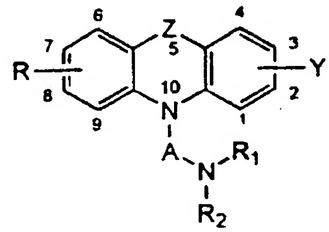
Compounds suitable for use in the methods of the invention include single enantiomers, racemates and geometric isomers of compounds having the structural skeletons of Formulas I, II, or III, and the structures and compounds described below. It further includes mixtures of enantiomers and geometric isomers of these structures and compounds.

Examples of drugs in the Phenothiazine family which are effective in blocking the appearance of paired helical filament epitopes include Promazine, Triflupromazine, Methotrimeprazine, Acetophenazine, Fluphenazine, Perphenzine, Prochloroperazine, Mesoridazine and Thoridazine. These and other similar phenothiazine compounds are described in U.S. Patent Nos. 2,921,069; 2,837,518; 2,860,138; 2,902,484; 3,194,733; 3,084,161; 2,519,886; 2,645,640; and 2,985,654, which are hereby incorporated by reference. Examples of drugs in the thioxanthene family that are effective in blocking the appearance of paired helical filament epitopes are Chlorprothixene and Thiothixene. Representative thioxanthenes are described in U.S. Patent Nos. 3,310,553 and 2,951,082, which also are hereby incorporated by reference.

Representative phenothiazine compounds useful in practicing the method of this invention are:

<u>U.S. Patent No. 2,921,069</u>

Compounds of the general formula:

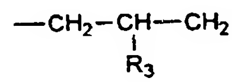


wherein Z is -S- or -SO-; Y is trifluoromethyl; R is hydrogen, halogen, trifluoromethyl, lower alkyl or lower alkoxyl; A represents a divalent, straight or branched aliphatic chain containing 2 to 6 carbon atoms; and R_1 and R_2 are either hydrogen, lower alkyl or divalent groups which together with the nitrogen to which they are attached form a five to seven-membered heterocyclic ring, such as pyrrolidinyl, piperazinyl, lower alkyl piperazinyl, piperidyl, thiomorpholinyl, morpholinyl or hexahydroazepinyl.

Advantageous compounds for use in this invention are represented by the above structural formula wherein: Z is -S-; Y is a trifluoromethyl in the 2 or 4 position; R is hydrogen, halogen, trifluoromethyl, lower alkyl or lower alkoxy in the 6 or 8 position; A is a divalent, straight or branched aliphatic chain containing 2 to 5 carbon atoms; and R_1 and R_2 represent either hydrogen, lower alkyl or divalent groups which together with the nitrogen to which they are attached form a five to six-membered heterocyclic ring, such as pyrrolidinyl, piperazinyl, lower alkyl piperazinyl, piperidyl, thiomorpholinyl or morpholinyl.

Still more advantageous compounds of this invention are represented by the above structural formula wherein: Z is -S-; Y is trifluoromethyl in the 2 or 4 position; R is hydrogen; A is either a divalent, straight aliphatic chain containing 2 to 4 carbon atoms or a divalent, branched aliphatic chain containing 2 to 5 carbon atoms; and R_1 and R_2 represent either hydrogen, lower alkyl or divalent groups which together with the nitrogen to which they are attached form a five to six-membered heterocyclic ring, such as pyrrolidinyl, piperazinyl, lower alkyl piperazinyl, piperidyl, thiomorpholinyl or morpholinyl.

The most preferred compounds are represented by the structural formula wherein: Z is -S-; Y is trifluoromethyl in the 2 position; R is hydrogen; A is a chain represented by the structure:



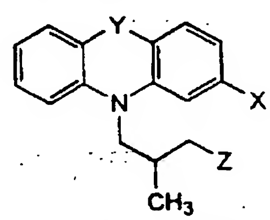
wherein: R_3 is hydrogen, methyl or ethyl; and R_1 and R_2 represent lower mono-or dialkyl or divalent groups which taken together with the nitrogen to which they are attached form a five to six-membered heterocyclic ring, such as pyrrolidinyl, piperazinyl, lower alkyl piperazinyl, piperidinyl, thiomorpholinyl or morpholinyl.

The terms lower alkyl or lower alkoxyl used above refer to aliphatic groups having not more than 4 carbon atoms and preferably not more than 2 carbon atoms, as indicated.

Methods for preparing these compounds are described in U.S. Patent No. 2,921,069.

U.S. Patent No. 2,837,518

Compounds of the general formula:

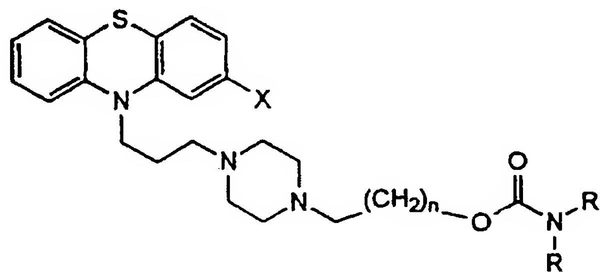


wherein Y is selected from the class consisting of a sulphur atom and the SO and SO_2 groups; X is selected from the class consisting of a hydrogen atom, a methyl, a methoxy, an ethyl and an ethoxy group; and Z is selected from the class consisting of the monomethylamino, monoethylamino, dimethylamino, diethylamino, pyrrolidino and piperidino groups.

Methods for preparing these compounds are described in U.S. Patent No. 2,837,518.

U.S. Patent No. 2,860,138

Compounds of the structural and general formula include:

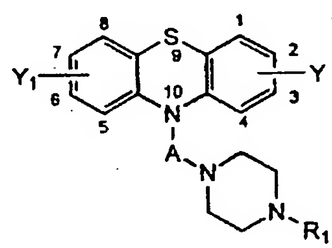


wherein X is a halogen atom having an atomic number greater than 9 but less than 53; R is a member of the group consisting of hydrogen and lower alkyl groups whose combined number of carbon atoms is less than 6; and n is a natural number less than 3.

Methods for preparing these compounds are described in U.S. Patent No. 2,860,138.

U.S. Patent No. 2,902,484

Compounds of the general formula:

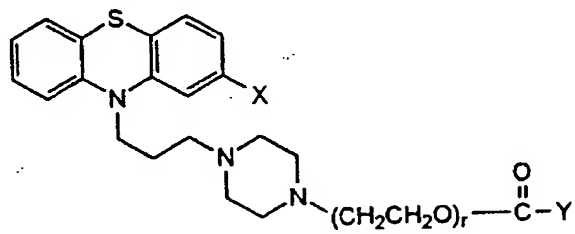


wherein A is a divalent saturated aliphatic hydrocarbon radical with a straight or branched chain containing from 2 to 6 carbon atoms; R_1 is a hydrogen atom, or a lower alkyl, aryl or arylalkyl group; Y and Y_1 represent a hydrogen or halogen atom, or a lower alkyl, alkoxy or aryl or aryloxy group, preferably (in the case of Y) in the 1- or 3- position. The phenothiazine ring may carry a substituent in the form a methyl group. It should be understood that the term "lower alkyl" means an alkyl group containing not more than 6 and preferably not more than 4 carbon atoms.

Methods for preparing these compounds are described in U.S. Patent No. 2,902,484.

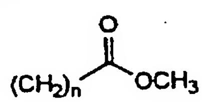
U.S. Patent No. 3,194,733

Compounds of the general formula:

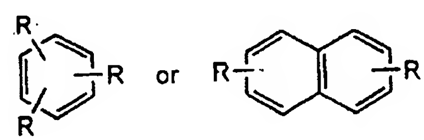


wherein r is 1 or 2; X is hydrogen, halogen (preferably chloro), trifluoromethyl, lower alkyl, lower alkoxy, lower alkanoyl, lower alkyl mercapto, trifluoromethylmercapto, and lower alkysulfonyl (preferably methylsulfonyl); and Y is higher alkyl, higher alkenyl, higher alkynyl, aryl, ω-carboalkoxy (higher alkyl) or diphenyl (hydroxymethyl). The terms "higher alkyl," "higher alkenyl" and "higher alkynyl" as employed herein include both straight and branched chain radicals of more than five carbon atoms.

The terms "ω-carboalkoxy (higher alkyl)" as employed herein, includes substituents derived from hydrocarbon carboxylic acids of more than 6 carbon atoms, and may be represented by the formula:



wherein n is a positive integer of more than 6 and is preferably a positive integer of from 7 to 12. The term "aryl" as employed herein includes substitutents derived from moncyclic and bicyclic aryl carboxylic acids, and may be substituted or unsubstituted and further may be represented by the formulae:



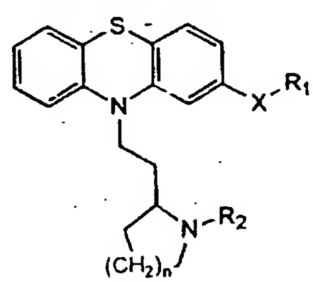
wherein each R is hydrogen, lower alkyl, lower alkoxy or halogen (e.g. chloro or bromo). R is preferably hydrogen, lower alkyl or lower alkoxy, and most preferably, is hydrogen or lower alkyl. Examples of the aryl carboxylic acids which may be employed include benzoic, o-toluic, 2,6-dimethylbenzoic, 2,6-dimethylanisic, o-bromobenzoic, o-chlorobenzoic, 2,6-dichlorobenzoic, naphthoic acid, dimethylnaphthoic acid, and other like acids.

The preferred compounds are those wherein X is chloro or trifluoromethyl and Y is a higher alkyl, higher alkenyl or higher alkynyl radical of from six to fourteen carbon atoms, lower alkyl- of lower alkoxy-substituted aryl, or ω-carboaloxy (higher alkyl) or less than 13 carbon atoms. Particularly preferred are those compounds wherein X is tifluoromethyl and Y is a higher alkyl radical of from nine to fourteen carbon atoms.

Methods for preparing these compounds are described in U.S. Patent No. 3,194,733.

U.S. Patent No. 3,084,161

Compounds of the general formula:

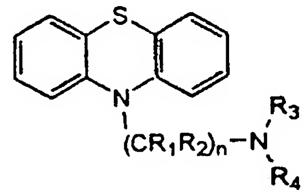


wherein X is S, SO, or SO_2; and each of R_1 and R_2 is a member selected from the group consisting of alkyl radicals containing from 1 to 4 carbon atoms; and n is an integer from 1 to 2.

Methods for preparing these compounds are described in U.S. Patent No. 3,084,161.

U.S. Patent No. 2,519,886

Compounds of the structural and general formula include:



wherein R_1 and R_2 are members of the class consisting of hydrogen atoms and alkyl groups (for example, methyl, ethyl and propyl); R_3 and R_4 represent alkyl groups (for example, methyl, ethyl, propyl, butyl); and n represents an integer greater than 1 (n may conveniently represent, for example, 2, 3, 4 or 5). The benzene nuclei may be substituted by alkyl or alkoxy groups. It should be understood that the designation:

$$(CR_1R_2)_n$$

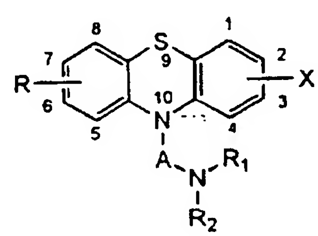
relates to straight aliphatic chains in which the successive CR_1R_2 groups are identical, branched aliphatic chains in which successive CR_1R_2 groups are identical, and branched aliphatic chains in which successive CR_1R_2 groups may be different. For example, the CR_1R_2 designation includes the branched chain:

$$-CH_2C(CH_3)_2-.$$

Methods for preparing these compounds are described in U.S. Patent No. 2,519,886.

U.S. Patent No. 2,645,640

Compounds of the general formula:



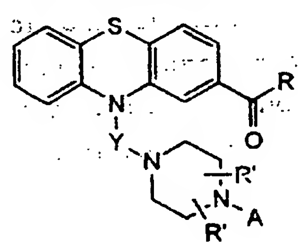
wherein R is a hydrogen, chlorine or bromine atom, or a methyl or methoxy group in the 6- or 8- position; X is either a chlorine or a bromine atom in the 1- of 3- position; A is a divalent, straight or branched aliphatic chain containing from 2 to 5 carbon atoms; and R_1 and R_2 are either individual methyl or ethyl groups or divalent groups which together with the adjacent nitrogen atom form a mononuclear heterocyclic ring.

Preferably A represents an alkylene group containing from 2 to 5 carbon atoms and R_1 and R_2 each represent a methyl or ethyl group or together represent the atoms necessary to complete a pyrrolidine, piperidine or morpholine nucleus.

Methods for preparing these compounds are described in U.S. Patent No. 2,645,640.

U.S. Patent No. 2,985,654

Compounds of the general formula:



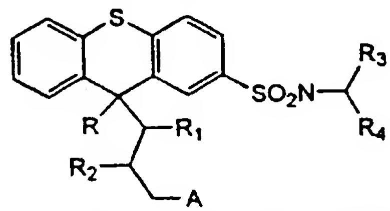
wherein Y is a bivalent hydrocarbon radical of from 2 to 5 carbon atoms; R is a lower alkyl group; R′ is a hydrogen or lower alkyl; and A is a member of the group consisting of saturated and unsaturated aliphatic hydrocarbon groups and hydroxyalkyl groups including ethers and esters of the hydroxy group. Typical examples of the group represented by R are methyl, ethyl and propyl. Representative of A are groups such as methyl, ethyl, allyl, propragyl, butenyl, 2-hydroxyethyl, 3-hydroxypropyl, 2-(2-hydroxyethoxy)-ethyl, 2-acetoxyethyl, 2-carbamoylethyl, and the like. Included in the group Y are ethylene, propylene, isopropylene, butylene, and the like.

Methods for preparing these compounds are described in U.S. Patent No. 2,985,654.

Representative thioxanthene compounds useful in practicing the methods of this invention are:

U.S. Patent No. 3,310,553

Compounds of the general formula:

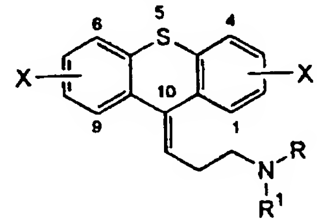


and the acid-addition salts thereof, wherein: R is hydrogen or, when taken together with R_1, forms a single bond; R_1 is selected from the group consisting of hydrogen and lower alkyl or, when taken together with R forms a single bond; R_2 is selected from the group consisting of hydrogen and lower alkyl; R_3 and R_4 when taken separately are selected from the group consisting of hydrogen and lower alkyl, and R_3 and R_4 when taken together with the nitrogen atom to which they are attached form a cyclic member selected from the group consisting of pyrrolidino, piperidino, morpholino and 4-lower alkylpiperazino; and A is selected from the group consisting of dialkylamino, 4-alkylpiperazinyl, 4-hydroxyalkylpiperazinyl, 4-acyloxyalkylpiperazinyl, 4-carbamylalkylpiperazinyl, 4-monoalkylcarbamylalkylpiperazinyl, 4-dialkylcarbamylalkylpiperazinyl, 4-alkoxyalkylpiperazinyl, 4-aryloxyalkylpiperazinyl, 4-alkoxyalkylpiperazinyl, 4-aryloxyalkylpiperazinyl,4-hydroxyalkyloxyalkylpiperazinyl, 4-acylalkylpiperazinyl, 4-aroylalkylpiperazinyl, 4-carboalkoxypiperazinyl, 4-carbamylpiperazinyl, 4-monoalkylcarbamylpiperazinyl,4-dialkylcarbamylpiperazinyl, 4-acylpiperazinyl, 4-aroylpiperazinyl, 4-alkylsufonylpiperazinyl and 4-dialkylsufamylpiperazinyl, said alkyl and acyl-groups containing up to about 4 carbon atoms.

Methods for preparing these compounds are described in U.S. Patent No.3,310,553.

U.S. Patent No. 2,951,082

Compounds of the general formula:



wherein X is a hydrogen, a halogen selected from the group consisting of chlorine, bromine, fluorine and iodine, a straight or branched chain alkyl radical having from 1 to about 4 carbon atoms, and a straight or branched chain alkoxy radical having with from 1 to 4 carbon atoms; and

is a tertiary amino group which advantageously is a dilower-alkylamino, 1-piperidyl, 1-pyrrolidyl or 4-morpholinyl group. One or more of the hydrogens attached to the trimethylene side chain may be substituted by a lower alkyl group provided the total number of carbons in all substituent alkyl groups does not exceed 4. When one or more of the trimethylene hydrogens is substituted by an alkyl group, one of the alkyl substituents can be linked with R^1 to form a heterocyclic ring including the nitrogen atom.

Methods for preparing these compounds are described in U.S. Patent No. 2,951,082.

This invention also includes salts of the above defined bases formed with non-toxic organic and inorganic acids. Such salts are easily prepared by methods known to the art. The base is reacted with either the calculated amount of organic or inorganic acid in aqueous miscible solvent, such as acetone or ethanol, with isolation of the salt by concentration and cooling or an excess of the acid in aqueous immiscible solvent, such as thyl ether or chloroform, with the desired salt separating directly. Exemplary of such organic salts are those with maleic, enesalicyclic, methysulfonic,

ethansesulfonic, acetic, propionic, artaric, salicyclic, citric, gluconic, lactic, malic, mandelic, cinnamic, citraconic, aspartic, stearic, palmitic, itaconic, glycolic, p-aminobenzoic, glutamic, benzane sulfonic and theophylline acetic acids as well as with the 8-halotheophyllines, for example, 8-chlorotheophylline and 8-bromotheophylline. Exemplary of such inorganic salts are those with hydrochloric, hydrobromic, sufuric, sulfamic, phosphoric and nitric acids. Of course these salts may also be prepared by the classical method of dobule decomposition of appropriate salts which is well known in the art. Those skilled in the art would recognize that this invention includes the use of enantiomers, homologs and isomers of the above-identified compounds.

These compounds may be formulated into pharmaceutical dosage forms by well recognized methods.

The compounds described above may be administered orally, topically, parenterally, by inhalation or spray or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and vehicles. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intrasternal injection or infusion techniques. In addition, there is provided a pharmaceutical formulation comprising one of the above compounds and a pharmaceutically acceptable carrier. One or more of these compounds may be present in association with one or more non-toxic pharmaceutically acceptable carriers and/or diluents and/or adjuvants and if desired other active ingredients. The pharmaceutical compositions containing these compounds may be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs.

Compositions intended for oral use may be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions may contain one or more agents selected from the group consisting

of sweetening agents, flavoring agents, coloring agents and preserving agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients which are suitable for the manufacture of tablets. These excipients may be for example, inert diluents, such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and dinintegrating agents, for example, corn starch, or alginic acid; binding agents, for example starch, gelatin or acacia, and lubricating agents, for example magnesium stearate, stearic acid or talc. The tablets may be uncoated or they may be coated by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monosterate or glyceryl distearate may be employed.

Formulations for oral use may also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example peanut oil, liquid paraffin or olive oil.

Aqueous suspensions contain the active materials in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example sodium carboxymethylcellulose, methylcellulose, hydropropylmethylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents may be a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylen oxide with long chain aliphatic alcohols, for example heptadecaethylene-oxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or

condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions may also contain one or more preservatives, for example ethyl, or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents, and one or more sweetening agents, such as sucrose or saccharin.

Oily suspensions may be formulated by suspending the active ingredients in a vegetable oil, olive oil, sesame oil or coconut oil, or in a mineral such as liquid paraffin. The oily suspensions may contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. Sweetening agents such as those set forth above, and flavoring agents may be added to provide palatable oral preparations. These compositions may be preserved by the addition of an anti-oxidant such as ascorbic acid.

Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those already mentioned above. Additional excipients, for example sweetening, flavoring and coloring agents, may also be present.

Pharmaceutical compositions of the invention may also be in the form of oil-in-water emulsions. The oily phase may be a vegetable oil, for example olive oil or arachis oil, or a mineral oil, for example liquid paraffin or mixtures of these. Suitable emulsifying agents may be naturally-occurring gums, for example gum acacia or gum tragacanth, naturally-occurring phosphatides, for example soy bean, lecithin, and esters or partial esters derived from fatty acids and hexitol, anhydrides, for example sorbitan monoleate, and condensation products of the said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monoleate. The emulsions may also contain sweetening and flavoring agents.

Syrups and elixirs may be formulated with sweetening agents, for example glycerol, propylene glycol, sorbitol or sucrose. Such formulations may also contain a demulcent, a preservative and flavoring and coloring agents. The pharmaceutical compositions may be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents which have been mentioned above. The sterile injectable preparation may also be sterile injectable solution or suspension in a non-toxic parentally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono-or-diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

The above described compounds may also be administered in the form of suppositories for rectal administration of the drug. These compositions can be prepared by mixing the drug with a suitable non-irritating excipient which is solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Such materials are cocoa butter and polyethylene glycols.

The above-identified compounds may be administered parenterally in a sterile medium. The drug, depending on the vehicle and concentration used, can either be suspended or dissolved in the vehicle. Advantageously, adjuvants such as local anaesthetic, preservative and buffering agents can be dissolved in the vehicle.

Dosage levels of the order of from about 0.1 mg to about 2500 mg per day are useful in the treatment of the above-indicated. The amount of active ingredient that may be combined with the carrier materials to produce a single dosage

form will vary depending upon the host treated and the particular mode of administration. Dosage unit forms will generally contain between from about 1 mg to about 500 mg of an active ingredient.

It will be understood, however, that the specific level for any particular patient will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration and rate of excretion.

Preferred compounds are:

Compound	Name
Promazine	N,N-Dimethyl-10H-phenothiazine-10-propanamine
Trifluoperazine	10-[3-[4-Methyl-1-piperazinyl]-propyl]-2-(triflurometbyl)-10H-phenothiazine
Triflupromazine	N,N-Dimethyl-2-(trifluoromethyl)-10H- phenothiazine-10-propanamine
Chlorpromazine	2-Chloro-N,N-dimethyl-10H-phenothiazine-10-propanamine
Methotrimeprazine	2-Methoxy-N,N,ß-trimethyl-10H-phenothiazine-10-propanamine
Acetophenazine	1-[10-[3-[4-(2-Hydroxyethyl)-1 piperazinyl]propyl]-10H-phenothiazine- 2-yl]ethanone
Fluphenazine	4-[3-[2-(Trifluoromethyl)-10H-phenothiazin-10-yl]propyl]-1-piperazineethanol
Perphenazine	4-[3-(2-Chloro-10H-phenothiazine-10-yl)propyl]-1-piperazineethanol

Prochlorperazine	2-Chloro-10[3-4-methyl-1-piperazinyl)propyl]-10H-phenothiazine
Mesoridazine	10-[2-(1-Methyl-2-piperidinyl)ethyl]-2-(methylsulfinyl)-10H-phenothiazine
Thioridazine	10-[2-(1-Methyl-2-piperidinyl)ethyl]-2- (methylthio)-10H-phenothiazine
Chlorprothixene	3-(2Chloro-9H-thioxanthen-9-ylidene)-N,N-dimethyl-1-propanamine
Thiothixene	N,N-Dimethyl-9-[3-(4-methyl-1-piperazinyl)propylidene]thioxanthene-2-sulfonamide

The present invention is described in the following Experimental Details Section, which sets forth specific examples to aid in an understanding of the invention, and should not be construed to limit in any way the invention as defined in the claims which follow thereafter.

Experimental Details Section

I. In Vitro Model System

A. Materials and Methods

Reagents and Media. Okadaic acid (OKA) was obtained from Moana Biochemical (Honolulu), anisomycin was obtained from Sigma, and alkaline phosphatase was obtained from Boehringer Mannheim. ^{32}P-orthophosphoric acid was purchased from NEN, and ^{35}S-1-methionine was purchased from ICN Biomedicals. RPMI 1640 medium for routine growth of MSN cells, and phosphate-free and methionine-free RPMI media were obtained from GIBCO BRL.

Antibodies. Antibodies T3P and tau46 were provided by Dr. Virginia Lee of the University of Pennsylvania. Monoclonal

antibodies 188 and 147 were raised against highly enriched pair helical filament preparations and were provided by Abbott Laboratories. E11 is a polyclonal antiserum against the human exon 11 peptide of tau and was provided by Dr. Hana Kseizak-Reding of Albert Einstein College of Medicine of Yeshiva University. Tau-1 was obtained from Dr. Lester Binder of UAB.

Cell Culture. The SMS-MSM-A human neuroblastoma cell line (MSN) was cloned from a population of SMS-MSN cells obtained from the laboratory of Dr. June Biedler (Sloan-Kettering, New York, NY), and was deposited with the American Type Culture Collection in Rockville, Maryland on February 2, 1993, and catalogued as ATCC # CRL 11253. The deposited human neuroblastoma cell line was tested for viability and determined viable on February 9, 1993. The neuroblastoma cells were grown in T75 flasks in RPMI 1640 medium supplemented with 15% fetal calf serum.

Oka Treatment of MSN Cells. OkA was dissolved in dimethyl sulfoxide (DMSO) and aliquots were added to Krebs-Ringer-phosphate buffer (KRP) containing 0.122 M NaCl, 4.9 mM KCl, 1.3 mM $CaCl_2$, 1.6 mM Na_2HPO_4, and 10 mM dextrose (pH 7.4) to produce the required concentration. Control samples were incubated with similar aliquots of the DMSO vehicle, and the final concentration of DMSO was maintained below 0.1%. Before incubation, the growth medium was aspirated off from each flask, and replaced with KRP containing either vehicle or OkA. The flasks were incubated at 37°C for the appropriate durations.

For experiments involving cell lysates, MSN cells were pelleted, resuspended in 250 µl cold Tris-buffered saline (TBS) containing 4 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM EGTA, and 25 µM leupeptin, and disrupted by sonicating with a 2 second pulse. Okadaic acid or vehicle (control) were added to the appropriate samples on ice and incubation was initiated at 37°C.

Preparation of Samples for SDS-PAGE. MSN cells were mechanically dislodged by vigorously shaking the flasks, and the entire contents were transferred to 15 ml conical tubes placed on ice. The flasks were rinsed with cold Tris buffered saline (TBS), and the entire cell suspension was centrifuged at 3000 g for 5 minutes in order to collect the cells. The cell pellets were resuspended in 250 μl TBS containing 4 mM PMSF and 2 mM EGTA, and boiled for 10 minutes. The heat stable proteins were assayed for protein by a micro BioRad procedure.

Electrophoresis and Blotting. Aliquots of heat-treated supernatant containing 20 μg protein were boiled with electrophoresis sample buffer and loaded onto mini 10% SDS-PAGE gels. The gels were electrophoretically transferred to nitrocellulose membrane and immunolabelled as previously described (Vincent, I. and Davies, P. PNAS USA 87:4840-4844 (1990)).

Protein Synthesis Inhibition. MSN cells were preincubated at 37°C for 1 hour with KRP in the absence or presence of 100 mM anisomycin. At the end of this time, 1 μM OkA was introduced into the appropriate flasks, and the samples incubated a further 90 minutes. Samples were then prepared for Western blot analysis according to routine protocol.

Alkaline Phosphatase Treatment. Nitrocellulose blotted protein samples were incubated at 37°C for 24 hours with 20 units per ml of alkaline phosphatase in pH 8 buffer containing 10 mM PMSF. Blots were subsequently used for Western blot analysis.

^{32}P-labeling of MSN Cells. Confluent layers of MSN cells were washed by incubation for 5 minutes at 37°C with RPMI 1640 medium lacking phosphate. Cells were labelled according to two protocols. A) Pulse-chase. Two milliliters of fresh phosphate-free medium was added to each flask, spiked with 2.5

mCi of ^{32}P-orthophosphoric acid and incubated at 37°C for 1 hour. At the end of this time the cells were washed once with KRP, and then further incubated for 90 minutes at 37°C with fresh KRP in the absence or presence of 1 μM OkA. B) Pulse. Cells washed with phosphate-free buffer were preincubated for 30 minutes with fresh phosphate-free medium containing 2.5 mCi per ml of ^{32}P-orthophosphoric acid. Following this time, 1 μM OkA was added to one flask and the cells were incubated for a further 90 minutes. With either protocol, heat-stable preparations were then prepared as described above, and 35 μg of protein of the heat-stable supernatants was clarified with respect to tau by treatment with 2.5% perchloric acid (PCA) for 10 minutes on ice (Lindwall, G. and Cole, R.D. J. Biol. Chem. 259:12241-12245 (1984)). The samples were centrifuged, the PCA-soluble protein was methanol precipitated and used for SDS-PAGE, together with sample buffer.

[^{35}S]Methionine Labeling of MSN Cells. After washing with methionine-free RPMI medium, MSN cells were preincubated overnight at 37°C with similar medium containing 200 μCi per ml of ^{35}S-l-methionine. The flasks were rinsed with cold KRP containing 2 mM unlabelled methionine, and chased for 90 minutes with medium containing methionine. Some samples were chased in the presence of 1 μM OkA either without or with 100 μM anisomycin. Thirty-five micrograms of the heat-stable preparations were subjected to PCA treatment and methanol precipitation as above.

B. Results

Time Course for OkA Treatment. The human neuroblastoma cell line SMS-MSN (MSN) expresses tau protein as a doublet of apparent molecular weights 57 and 60 kDa. These proteins were detected with antibodies Alz-50, Tau-1, PHF-1, NP8 and T3P when 20 μg of heat-stable protein was utilized for SDS-PAGE and Western blot analysis (0 minute control, Figure 1). As a first step in investigating the role of protein phosphatases on these various epitopes, MSN cells with OkA, a cell-

permeable protein phosphatase inhibitor (Bialojan, C. and Takai, A. J. Biochem. 256:283-290 (1988)). The monoclonal antibody 188 which recognizes primary sequence in tau was used as a negative control in these experiments. While the 0.1% dimethyl sulfoxide (DMSO) vehicle produced no changes (see 4 hour control, Figure 1), a 30 minute exposure to 1 μM OkA resulted in a marked induction of the phosphorylated tau epitopes, PHF-1, NP8, and T3P. In addition, OkA treatment induced the appearance of PHF-1, NP8, and T3P epitopes on several higher molecular weight proteins. Both PH1 and T3P reactivities were observed with a 68-kDa protein (Figure 1). In addition, PHF-1 positive bands at 85, 120, and 180 kDa, a T3P-positive band at 110 kDa, and several NP8 positive bands, including a major band at 160 kDa, became apparent. The isolated occurence of these epitopes on higher molecular weight proteins in the preparation suggests that they are independent epitopes. Unexpectedly, the treatment of MSN cells with OkA also concurrently increased the reactivity of tau with Alz-50 (Figure 1).

In contrast to the above increases in immunoreactivities, incubation with OkA for durations over 30 minutes resulted in an obliteration of Tau-1 reactivity with the proteins (Figure 1). These various OkA-induced alterations in immunoreactivity of tau were accompanied by a significant decrease in the electrophoretic mobility of the proteins on SDS-PAGE. This effect was indicated by a 2-3 kDa upward shift in the apparent molecular mass of tau, a feature characteristic of hyperphosphorylated tau proteins and PHFs (Baudier, J. and Cole, D.R. J. Biol. Chem. 262:17577-17583 (1987)).

Dose-Response Curves for OkA Treatment. Using the differential sensitivity of the various cellular protein phosphatases to OkA, MSN cells were exposed to different concentrations of drug to identify the enzymes(s) that regulate the production of specific tau epitopes. A concentration of 50 nM OkA produced a considerable activation

in immunoreactivities of Alz-50, 188, PHF-1, NP8, and T3P with tau (Figure 2). At this concentration, a decrease in electrophoretic mobility of tau also was apparent, and with higher concentrations of OkA the mobility of the proteins declined even further. Although some reduction in Tau-1 immunoreactivity was observed at 50 nM OkA, a concentration of 1 μM was required to abolish reactivity completely with the antibody (Figure 2). As with the time dependence, higher concentrations of OkA also induced immunoreactivity of numerous higher-molecular-weight proteins with PHF-1, NP8, and T3P. The 68 kDa protein was observed with PHF-1, NP8, and T3P from 50 nM OkA upward, and was also labeled with 188 (Figure 2). Additional proteins bearing epitopes reactive with PHF-1, NP8 and T3P also were evident.

Effects of Protein Synthesis Inhibition. In view of the overall induction of tau immunoreactivity following OkA treatment, its dependence upon de novo protein synthesis was examined. Figure 3 shows the effects of 100 μM anisomycin, a protein synthesis inhibitor, on the OkA-induced effects on tau. Consistent with their rapidity, the increases in immunoractivity of Alz-50, 188, PHF-1, NP8, or T3P and the shift in molecular weight do not require protein synthesis. Moreover, the disappearance of Tau-1 immunoreactivity that occurred as a result of OkA treatment was also observed in the presence of anisomycin (Figure 3).

Treatment of MSN Cell Lysates With OkA: Effects of Alkaline Phosphatase. The importance of cellular integrity in the OkA-induced alterations in tau immunoreactivity was assessed by sonicating MSN cells and exposing the resulting cell lysates to OkA. When the cell lysates were incubated under control conditions with KRP, immunoreactivity of PHF-1, NP8, and T3P with tau was rapidly abolished while the proteins retained retained reactivity with Alz-50, Tau-1, and 188 (Figure 4, (-)). A parallel reduction in apparent molecular mass of tau also was observed, with resolution into two major bands of 50

and 54 kDa. These alterations were observed in the presence of the protease inhibitors PMSF, leupeptin, and EGTA. When 1 μM OkA was introduced into the incubation, the disappearance of PHF-1, NP8, and T3P immunoreactivities and the decrease in apparent molecular weight of tau were blocked (Figure 4, (-)), indicating that these effects were mediated by dephosphorylation rather than proteolysis. Thus, in this broken-cell paradigm, OkA was effective in inhibiting the nonspecific effects of protein phosphatases, but was ineffective in activating immunoreactivity of tau or increasing its molecular weight. It is possible that ongoing protein phosphorylation is interrupted in cell lysates relative to intact cell preparations, and may account for the lack of activation of tau immunoreactivity and increase in molecular weight.

As an additional means of establishing that the observed changes in tau immunoreactivity were due to phosphorylation, duplicate blots of the above samples were incubated with alkaline phosphatase. It was found that subsequent reaction of PHF-1, NP8, and T3P with all proteins was abolished following phosphatase treatment (Figure 4, (+)). As is consistent with the nature of the Tau-1 epitope, a similar treatment results in enhancement of Tau-1 immunoreactivity. On the other hand, alkaline phosphatase treatment of the nitrocellulose blots did not influence Alz-50 and 188 immunoreactivities.

<u>^{32}P-Labeling of Tau</u>. To demonstrate definitively that OkA influences tau immunoreactivity as a result of increased phosphorylation, MSN cells were labelled with ^{32}P-orthophosphate according to two independent protocols. In the first protocol, the cells were pulse-labeled and subsequently incubated with KRP with or without OkA (Figure 5A). The presence of OkA in the chase produced a substantial accumulation of label in tau in comparison with cells chased with KRP alone. The corresponding immunoblot reveals that Alz-50 immunoreactivity with tau was barely detected when the

label was lost in the chase, but was greatly enhanced when incorporated phosphate was retained in the presence of OkA. In the second protocol, the pulse method, cells were exposed to OkA in the presence of ^{32}P-orthophosphate as a means of ascertaining the extent of phosphorylation of tau during the routine 90 minute OkA treatment. As shown in Figure 5B, although the degree of labelling was remarkably greater when OkA was present relative to the control, the overall incorporation of phosphate was also greater than that of the pulse-chase samples. These data illustrate the rapid turnover rate of phosphate in MSN cell tau.

[^{35}S]Methionine Labeling of Tau. Because of the increased immunoreactivity of non-phosphorylated epitopes (Alz-50 and 188) in tau produced by OkA treatment, it was reasoned that phosphorylation might lead to accumulation of tau protein by conferring resistance to proteolytic cleavage. With this in mind, the effects of OkA on the turnover of tau protein was examined. MSN cells were metabolically pulsed with isotopically labeled methionine and subsequently subjected to a chase with unlabeled methionine, in the absence or presence of OkA. In the absence of OkA, 35S-label and Alz-50 immunoreactivity of tau were rapidly lost (Figure 6). In the presence of OkA, both the loss of label and Alz-50 immunoractivity were prevented, indicating that degradation of tau is inhibited when the protein is in a highly phosphorylated state. This effect persists when de novo protein synthesis is prevented by the inclusion of 100 μM anisomycin in the OkA chase (Figure 6).

C. Discussion

The above experiments demonstrate a marked induction of five independent antigenic determinants that have been described as characteristic of PHF in Alzheimer's Disease. In addition, as is observed with PHF protein, the apparent molecular weights of tau is significantly increased. These alterations were produced as a result of manipulations in

serine-threonine protein phosphatase activity induced by OkA. However, since OkA-induced changes in immunoactivity do not always coincide with changes in SDS-gel mobility of tau (see Figure 2), the increased phosphorylation at the PHF-1, NP8, T3P and Tau-1 epitopes is not responsible for the increase in apparent molecular weight of tau. The appearance of 68 kDa protein with immunoreactivity towards PHF-1, NP8 and T3P (see Figures 1, 2 and 3) corresponds to an increase in stability of another normally transient isoform of tau. This protein is occasionally detected with Alz-50 or 188 (see Figures 5 and 6). The identity of the additional higher molecular weight proteins recognized by PHF-1, NP8 and T3P following OkA treatment is not known (see Figures 1, 2 and 3). Although they may represent related cytoskeletal proteins by virtue of their stability to boiling, they do not contain tau protein since each was positive with only one of the above antibodies, and none were recognized by monoclonal antibodies Alz-50 or 188.

The resistance of Alz-50 immunoreactivity to phosphatases endogenous to cell lysate preparations and to exogenous phosphatase treatment of blots (see Figure 4) reinforce the conclusion that OkA causes an increase in Alz-50 immunoreactivity. The concomitant activation of 188 immunoreactivity and at least three additional antibodies reactive with primary sequence determinants in addition to the increase in Alz-50 immunoreactivity predict an overall increase in the amount of tau protein in the neuroblastoma cells. The rapidity of these effects and their persistence in the presence of protein synthesis inhibitors preclude the possibility that they result from <u>de novo</u> protein synthesis. An alternate explanation for the general increase in tau immunoreactivity is reduced degradation. The ability of OkA to prevent loss of ^{35}S-methionine label in previously synthesized tau protein, in contrast to the rapid loss of label observed in the absence of OkA, supports this idea. Further, confirmation comes from the results of accumulation

of prelabelled tau in the presence of OkA plus the protein synthesis inhibitor anisomycin.

The importance of serine-threonine phosphatases in the formation of AD specific epitopes is underscored by the finding that the permeant tyrosine phosphatase inhibitors, vanadate and genstein are without effect. Thus, the decoration of Alzheimer's Disease neurofibrillary tangles and neuritic plaques by an anti-phosphotyrosine antibody correlates with an as yet unidentified epitope, or may be non-specific.

Since protein phosphatases display differential sensitivities to OkA, the concentration of inhibitor required for a given effect may be indicative of the specific enzyme involved. The IC-50 for phosphatase 1 is 0.1-0.5 mM, phosphatase 2A is 1 nM, and the IC-50 for phosphatase 2B (i.e., the Ca^{2+}/CAM-dependent phosphatase, Calcineurin) is 10 mM, whereas phosphatase 2C (i.e., the Mg^{2+}-dependent phosphatase) is unaffected. Optimal inductions in PHF-1, NP8, and T3P immunoreactivities occur at 50 nM, a concentration 50-fold that of the IC-50 for phosphatase 2A, making it likely that phosphatase 2A plays an important role in turnover of these epitopes. On the other hand, since this concentration is 2-10 fold less than the IC-50 for phosphatase 2B, it is unlikely that either of these phosphatases play role in regulation of these epitopes. Phosphorylation at the Tau-1 site is favored at or over 1 μM, although some inhibition in Tau-1 immunoreactivity is observed at 50 nM OkA. Thus, it is more likely that phosphatase 1 is involved in turnover of the phosphatase at the Tau-1 site, although a role for phosphatase 2A cannot be excluded.

The decrease in electrophoretic mobility of tau is first evident at 50 nM OkA, and it becomes increasingly evident with increasing concentrations of the drug. It is possible that the electrophoretic behavior of tau is dependent upon phosphorylation at more than one site, thereby implicating several protein phosphatases. A recent study with

human temporal lobe slices illustrates the importance of calcineurin in electrophoretic mobility of tau (Harris, et al. Ann. Neurol. 33:77-87 (1992)). On the other hand, previous *in vitro* studies have implicated phosphatase 2A in SDS-PAGE mobility of Tau (Yamamoto, et al. J. Neurochem. 55:683-690 (1990)). The suggested role of phosphatase 2A in the formation of the PHF-1, T3P and NP8 epitopes in MSN cells indicates that this phosphatase is crucial in determining the phosphorylation state of both normal and paired helical filament-like epitopes in tau, and may be an important enzyme in tau metabolism.

The observed insensitivity of hyper-phosphorylated tau protein to cellular proteases and their resulting accumulation might signify an early event leading to formation of insoluble filaments. Early detection of Alz-50 and Tau-1 immunoreactivities in neurons without apparent morphologic aberrations may correspond to a similar state in AD brain. There is evidence from *in vitro* studies that phosphorylation decreases the elasticity of tau (Hagestedt, et al. J. Cell Biol. 109:1643-1651 (1989) and decreases its solubility (Hanger, et al. Biochem. J. 275:99-104 (1991)). Moreover, purified phosphorylated forms of tau show less susceptibility to certain proteases than non-phosphorylated forms of tau (Litersky, J.M. and Johnson, G.V. J. Biol. Chem. 267:1563-1568 (1992)). Collectively, these data support the theory that hyper-phosphorylation renders tau protease-resistant and/or insoluble.

Within the last few years, paired helical filament-like epitopes have been discovered in human fetal brain tissue. it has also been found that tau from normal human fetal brain is positive for NP8 and T3P immunoreactivities. Hence, the above epitopes may be considered "normal." However, since they are not evident in normal adult brain, it appears that the enzymatic processes necessary for their formation are under developmental regulation. The reappearance of these phosphorylation sites in Alzheimer's Disease may therefore occur as a result of

deregulation of this enzymatic system, rather than as a result of an abnormal enzyme in Alzheimer's Disease brain. Alterations in intracellular localization and activities of the specific protein kinases and phosphatases that regulate each phosphorylation site in tau may serve as clues for aberrant post-translation processing of tau in Alzheimer's Disease. Although there is evidence of unusual activity and localization of protein kinase C and casein kinase II in Alzheimer's Disease, neither of these kinases has been implicated in formation of paired helical filament-like epitopes on tau.

II. <u>Immunoreactivity of Antibodies With Normal and Alzheimer's Disease Brain, and Treated and Untreated MSN Cells</u>

The immunoreactivity of monoclonal antibodies Alz-50, PHF-1, TG3, TG4, MC2, MC6, and MC15 with normal and Alzheimer's brain tissue, and with MSN cells before (MSN-) and after treatment (MSN+) with okadaic acid was examined, and the results are presented in Figure 7. The data from brain tissue are the mean values derived from studies of mid-temporal cortex from five normal cases and five cases of Alzheimer's Disease. Values from MSN cells are from a single experimental series in which 1 micromolar okadaic acid was added to triplicate MSN cell cultures for 90 minutes prior to harvest of the cells. Each sample (tissue or cell homogenate) was assayed at 8 dilutions in duplicate. The units on the Y axis are arbitrary units of immunoreactivity. In all cases, it is clear that okadaic acid produces increases in immunoreactivity in MSN cells, and that this increase is similar in extent to the difference between normal and Alzheimer's brain tissue.

III. <u>Effect of Trifluorperazine and Chlorpromazine On PHF Epitopes In MSN Cells</u>

In order to determine the effect of Trifluoperazine and Chlorpromazine on the PHF epitopes associated with Alzheimer's Disease, MSN-A cells expressing PHF epitopes were incubated with 100 μM Trifluoperazine (TFP), 100 μM

Chlorpromazine (CPZ) or 0.2% DMSO vehicle (control) for 2 hours at 37°C. The cells were isolated by centrifugation, boiled for 10 minutes and 25 μg protein from the resulting heat stable supernatants were loaded per lane on an SDS-PAGE gel. The gel was electrophoretically transferred to nitrocellulose membrane and then immunostained with PHF-1. As shown in Figure 8, both TFP and CPZ greatly decreased the production of paired PHF epitopes by the neuroblastoma cells. In contrast, the control shows that without the addition of TFP or CPZ, there was production of PHF epitopes by the neuroblastoma cells.

The effective concentrations appear to be in the range at which these drugs are present in the brain following chronic treatment of psychiatric patients (Svendsen, C.N. and Bird, E.D. Psychopharmacology 90:316-321 (1986)). These results suggest that patients who were chronically treated with phenothiazines or structurally related compounds would be protected from the development of PHF, and thus would have a low probability of developing Alzheimer's Disease.

IV. Study of Chronic Schizophrenics Treated With Chlorpromazine

Frontal and cortical brain tissue specimens were obtained at autopsy from chronic schizophrenic patients chronically treated with chlorpromazine and analyzed using the ALZ-EIA method as described (Ghanbari, H.A., et al. JAMA 263:2907 (1990)). Results are shown in Figures 9A (frontal (Broadman area 10)) and 9B (temporal (Broadman area 38)) regions. The brain tissue (post-mortem) from approximately the same number of (age matched) normal controls and Alzheimer's disease patients were also analyzed and are presented in the Figures as negative and positive controls (for normal and AD, respectively). As the data indicate, the samples from chlorpromazine treated patients (RX) had low PHF epitope levels similar to normal controls, whereas the PHF epitope levels in the AD group were clearly much higher. Furthermore, these results were consistent with

histopathological diagnosis of the brain specimens. Statistically, about 20-30% of the individuals in the RX age group would have developed AD and hence much higher PHF in the brain regions indicated. This evidence shows that chronic treatment of patients with chlorpromazine prevents PHF formation and AD. In a similar manner, the compounds which have in vitro activity like chlorpromazine are effective.

All publications and patents referenced hereinabove are incorporated by reference in their entirety.

Although the invention herein has been described with reference to particular embodiments, it is to be understood that these embodiments are merely illustrative of various aspects of the invention. Thus, it is to be understood that numerous modifications may be made in the illustrative embodiments and other arrangements may be devised without departing from the spirit and scope of the invention.

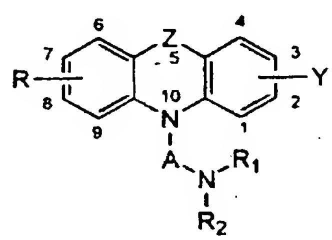
What is Claimed is:

1. A method for treating or preventing Alzheimer's Disease in a subject in need of such treatment or prevention which comprises administering an amount of a compound selected from the group consisting of phenothiazines, thioxanthenes, and the pharmaceutically acceptable salts thereof effective to prevent or diminish the accumulation of abnormally phosphorylated, paired helical filament epitopes associated with Alzheimer's Disease.

2. The method of Claim 1 wherein the compound is administered in a dosage range of about 1-2500 mg per day.

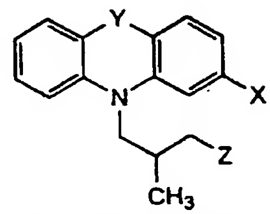
3. A method of Claim 1 wherein the compound is trifluoperazine or chlorpromazine.

4. The method according to Claim 1 wherein the compound has the formula:



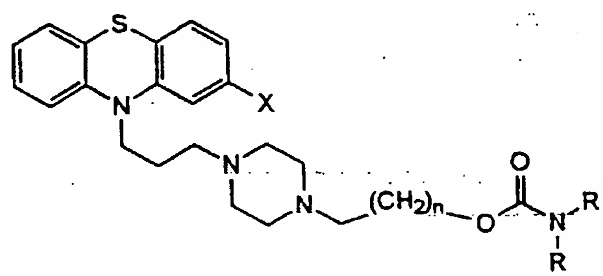
wherein Z is -S- or -SO-; Y is trifluoromethyl; R is hydrogen, halogen, trifluoromethyl, lower alkyl or lower alkoxyl; A is a divalent, straight or branched aliphatic chain containing 2 to 6 carbon atoms; and R_1 and R_2 are either hydrogen, lower alkyl or divalent groups which together with the nitrogen to which they are attached form a five to seven-membered heterocyclic ring, such as pyrrolidinyl, piperazinyl, lower alkyl piperazinyl, piperidyl, thiomorpholinyl, morpholinyl or hexahydroazepinyl; and pharmaceutically acceptable salts thereof.

5. The method according to Claim 1 wherein the compound has the formula:



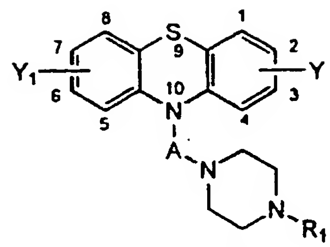
wherein Y is selected from the class consisting of a sulphur atom and SO and SO_2 groups; X is selected from the class consisting of a hydrogen atom, a methyl, a methoxy, an ethyl and an ethoxy group; and Z is selected from the class consisting of the monomethylamino, monoethylamino, dimethylamino, diethylamino, pyrrolidino and piperidino groups; and pharmaceutically acceptable salts thereof.

6. The method according to Claim 1 wherein the compound has the formula:



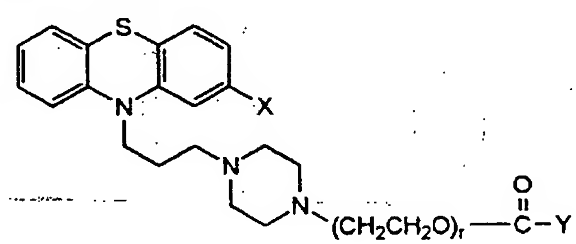
wherein X is a halogen atom having an atomic number greater than 9 but less than 53; R is a member of the group consisting of hydrogen and lower alkyl groups whose combined number of carbon atoms is less than 6; and n is a natural number less than 3; and pharmaceutically acceptable salts thereof.

7. The method according to Claim 1 wherein the compound has the formula:

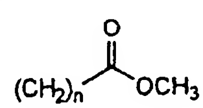


wherein A is a divalent saturated aliphatic hydrocarbon radical with a straight or branched chain containing from 2 to 6 carbon atoms; R_1 is a hydrogen atom, or a lower alkyl, an aryl or arylalkyl group; Y and Y_1 are a hydrogen or halogen atom, or a lower alkyl, alkoxy, aryl or aryloxy group, preferably (in the case of Y) in the 1- or 3-position, the phenothiazine ring may carry a substituent in the form of a methyl group; and pharmaceutically acceptable salts thereof.

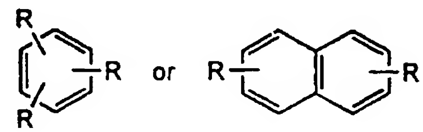
8. The method according to Claim 1 wherein the compound has the formula:



wherein r is 1 or 2; X is hydrogen, halogen, trifluoromethyl, lower alkyl, lower alkoxy, lower alkanoyl, lower alkyl mercapto, trifluoromethylmercapto, and lower alkysulfonyl; and Y is higher alkyl, higher alkenyl, higher alkynyl, aryl, ω-carboalkoxy (higher alkyl) or diphenyl (hydroxymethyl), wherein the "higher alkyl," "higher alkenyl" and "higher alkynyl" are both straight and branched chain of radicals of more than five carbon atoms and wherein the "ω-carboalkoxy (higher alkyl)" has substituents derived from hydrocarbon carboxylic acids of more than 6 carbon atoms, and may be represented by the formula:

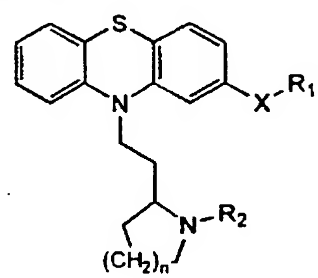


wherein n is a positive integer of 6 to 12 and wherein "aryl" may be substituted or unsubstituted and further may be represented by the formulae:



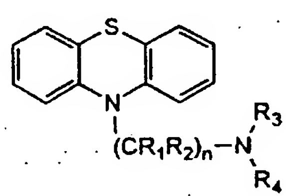
wherein each R is hydrogen, lower alkyl, lower alkoxy or halogen; and pharmaceutically acceptable salts thereof.

9. The method according to claim 1 wherein the compound has the formula:



wherein X is S, SO, or SO^2; and each of R_1 and R_2 is a member selected from the group consisting of alkyl radicals containing from 1 to 4 carbon atoms; and n is an integer from 1 to 2; and pharmaceutically acceptable salts thereof.

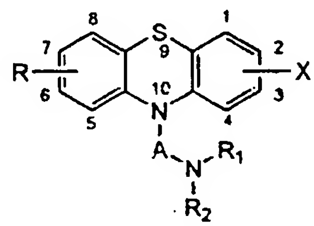
10. The method according to claim 1 wherein the compound has formula:



wherein R_1 and R_2 are members of the class consisting of hydrogen atoms and alkyl groups having 1 to 6 carbon atoms; R_3 and R_4 represent alkyl groups having 1 to 6 carbon atoms; and n is an integer greater than 1; and wherein the aromatic

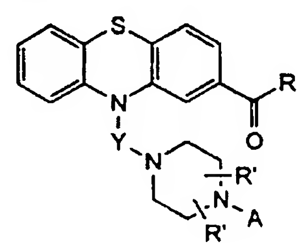
nuclei may be substituted by alkyl or alkoxy groups having 1 to 6 carbon atoms; and pharmaceutically acceptable salts thereof.

11. The method according to claim 1 wherein the compound has the formula:



wherein R is a hydrogen, chlorine or bromine atom, or a methyl or methoxy group in the 6- or 8-position; X is either a chlorine or a bromine atom in the 1- or 3-position; A is a divalent, straight or branched aliphatic chain containing from 2 to 5 carbon atoms; and R_1 and R_2 are either individual methyl or ethyl groups or divalent groups which together with the adjacent nitrogen atom form a mononuclear heterocyclic ring; and pharmaceutically acceptable salts thereof.

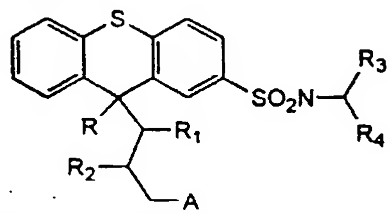
12. The method according to claim 1 wherein the compound has the formula:



wherein Y is a bivalent hydrocarbon radical of from 2 to 5 carbon atoms; R is a lower alkyl group, R' is a hydrogen or lower alkyl; and A is a member of the group consisting of saturated and unsaturated aliphatic hydrocarbon groups and

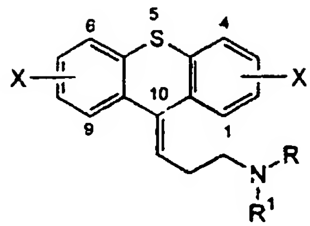
hydroxyalkyl groups including ethers and esters of the hydroxy group; and pharmaceutically acceptable salts thereof.

13. The method according to claim 1 wherein the compound has the formula:

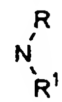


wherein R is hydrogen or, when taken together with R_1, forms a single bond; R_1 is selected from the group consisting of hydrogen and lower alkyl or, when taken together with R forms a single bond; R_2 is selected from the group consisting of hydrogen and lower alkyl; R_3 and R_4 when taken separately are selected from the group consisting of hydrogen and lower alkyl, and R_3 and R_4 when taken together with the nitrogen atom to which they are attached form a cyclic member selected from the group consisting of pyrrolidino, piperidino, morpholino and 4-lower alkylpiperazino; and A is selected from the group consisting of dialkylamino, 4-alkylpiperazinyl, 4-hydroxalkylpiperazinyl, 4-acyloxyalkylpiperazinyl, 4-carbamylalkylpiperazinyl, 4-monoalkylcarbamylalkylpiperazinyl, 4-dialkylcarbamylalkylpiperazinyl, 4-alkoxyalkylpiperazinyl, 4-aryloxyalkylpiperazinyl, 4-alkoxyalkylpiperazinyl, 4-aryloxyalkylpiperazinyl,4-hydroxyalkyloxyalkylpiperazinyl, 4-acylalkylpiperazinyl, 4-aroylalkylpiperazinyl, 4-carboalkoxypiperazinyl, 4-carbamylpiperazinyl, 4-monoalkylcarbamylpiperazinyl,4-dialkylcarbamylpiperazinyl, 4-acylpiperazinyl, 4-aroylpiperazinyl, 4-alkylsofonylpiperazinyl and 4-diakylsufamylpiperazinyl, said alkyl and acyl-groups containing up to about 4 carbon atoms; and pharmaceutically acceptable salts thereof.

14. The method according to claim 1 wherein the compound has the formula:



wherein X is a hydrogen, a halogen selected from the group consisting of chlorine, bromine, fluorine and iodine, a straight or branched chain alkyl radical having from 1 to about 4 carbon atoms, and a straight or branched alkoxy radical chain having from 1 to 4 carbon atoms; and



is a tertiary amino group wherein R and R^1 are lower alkyl having 1 to 4 carbom atoms, and R and R^1 together with N may form a heterocyclic ring; and pharmaceutically acceptable satls thereof.

15. The method according to claim 1 wherein the compound is selected from the group consisting of:

N,N-Dimethyl-10H-phenothiazine-10-propoanamine;

10-[3-4-[Methyl-1-piperazinyl]-propyl]-2-(trifluoromethyl)-10H-phenothiazine;

N,N-Dimethyl-2-(trifluoromethyl)-10H-phenothiazine-10-propanamine;

2-Chloro-N,N-dimethyl-10H-phenothiazine-10-propa;

2-Methoxy-N,N,B-trimethyl-10H-phenothiazine-10-propananmine;

1-[10-[3-[4-(2-Hydroxyethyl)-1-piperazinyl]propyl]-10H-phenothiazin-2-yl]ethanone;

4-[3-[2](Trifluoromethyl)-10H-phenothiazin-10-

yl]propyl}-1-piperazineethanol;

4-[3-(2-Chloro-10H-phenothiazin-10-yl)propyl]-1-piperazineethanol;

2-Chloro-10[3-4-methyl-1-piperazinyl)propyl]-10H phenothiazine;

10-[2-(1-Methyl-2-piperidinyl)ethyl]-2-(methylsufinyl)-10H-phenothiazine;

10-[2-(1-Methyl-2-piperidinyl)ethyl]-2-(methylthio)-10H-phenothiazine;

3-(2-Chloro-9H-thioxanthen-9-ylidene)-N,N-dimethyl-1-propananmine;

N,N-dimethyl-9-[3-(4-methyl-1-piperazinyl) propylidene]thioxanthene-2-sulfonamide;

and pharmaceutically acceptable salts thereof.

FIGURE 1

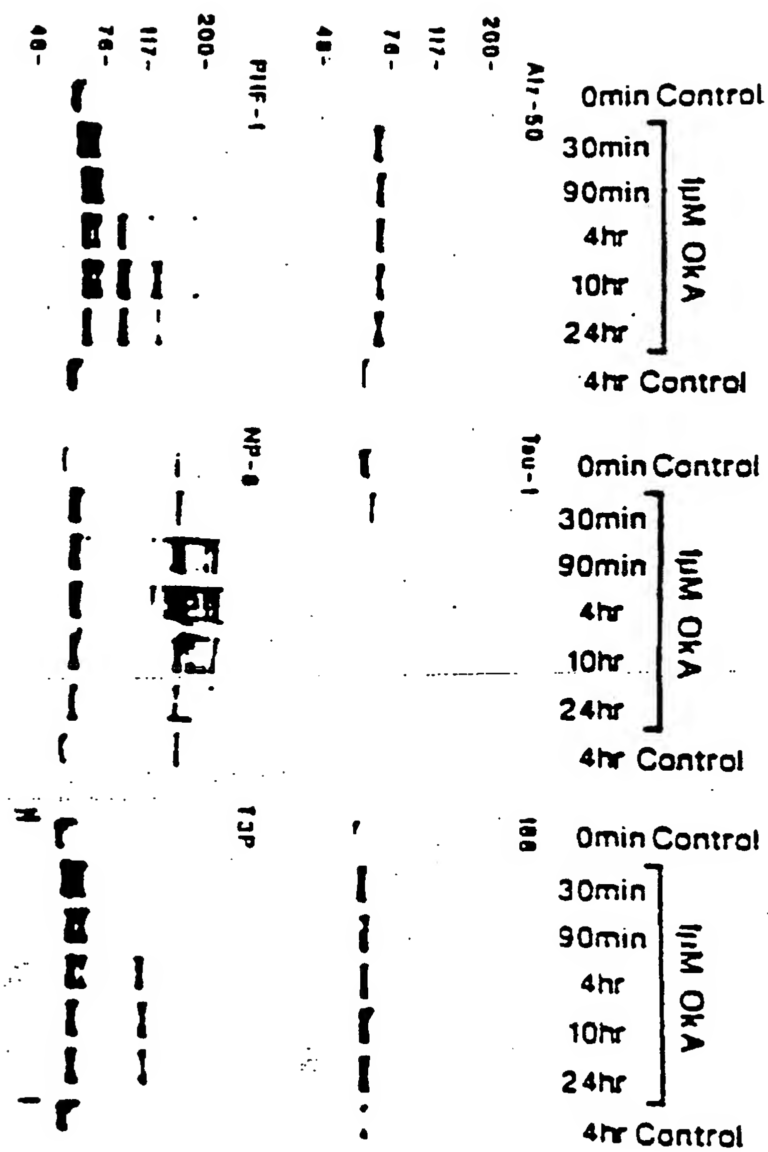

0min Control
30min
90min
4hr
10hr
24hr
4hr Control
1μM OkA
0min Control
30min
90min
4hr
10hr
24hr
4hr Control
1μM OkA
0min Control
30min
90min
4hr
10hr
24hr
4hr Control
1μM OkA

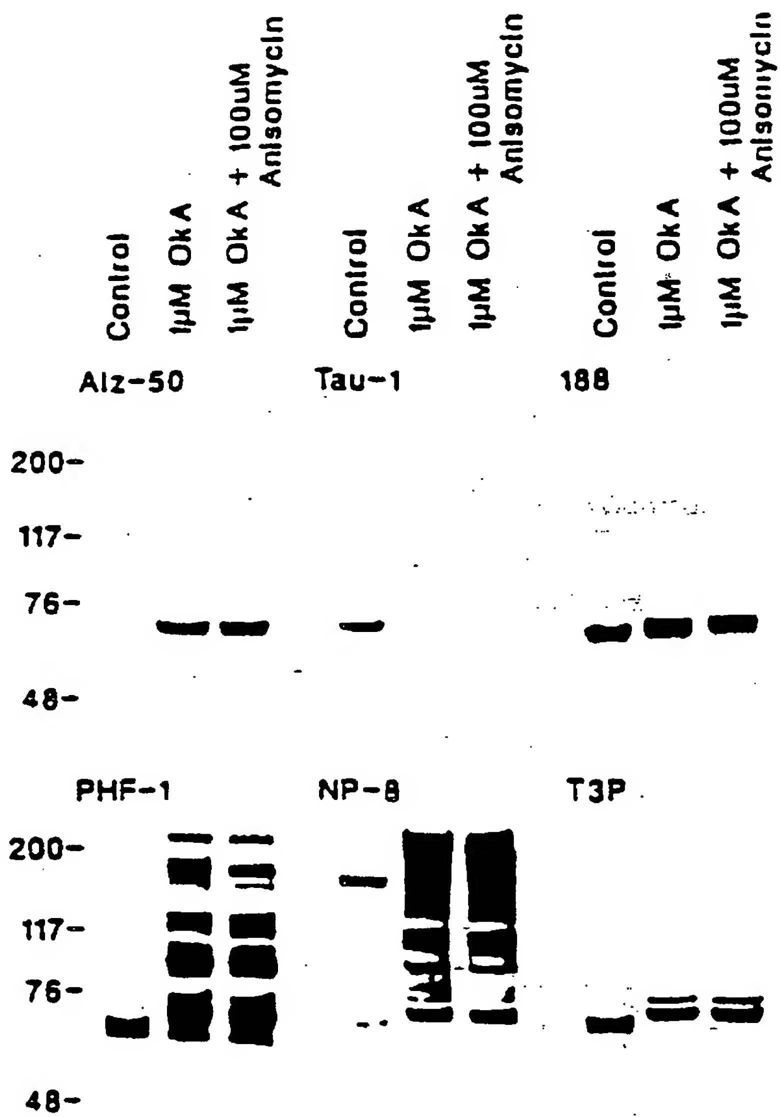
FIGURE 2


Alz-50
PHF-1
Tau-1
NP-8
188
T3P
200-
117-
76-
48-
Control
5nM
50nM
1µM
50µM

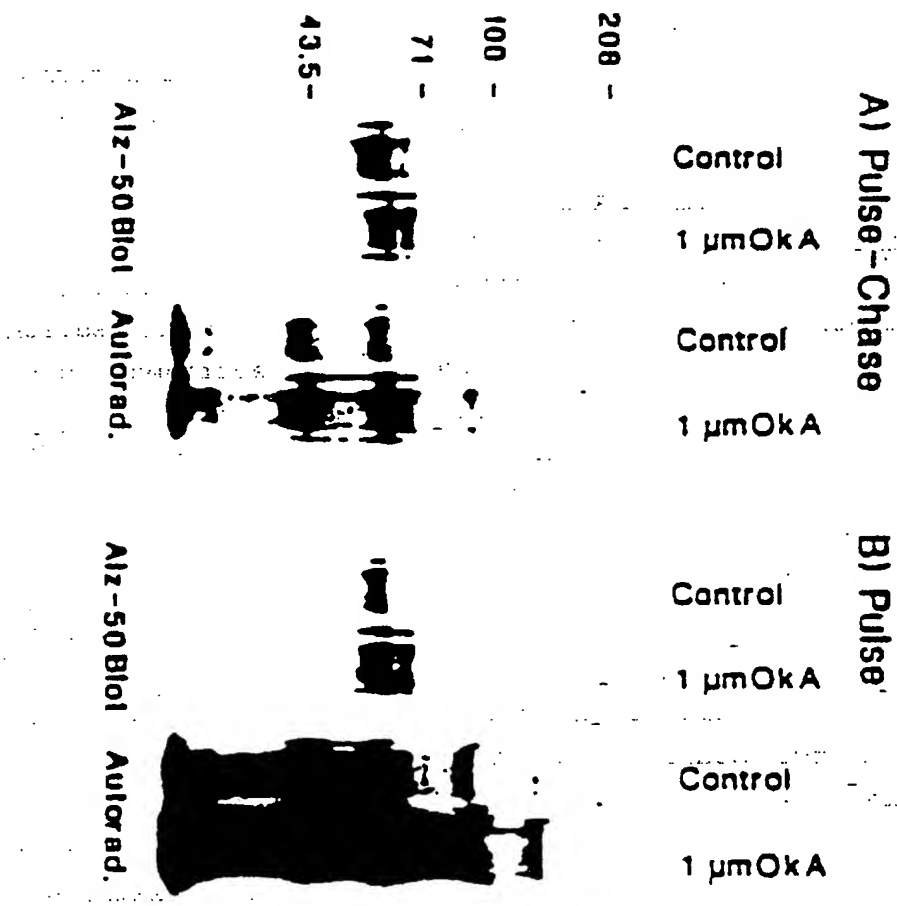
FIGURE 3

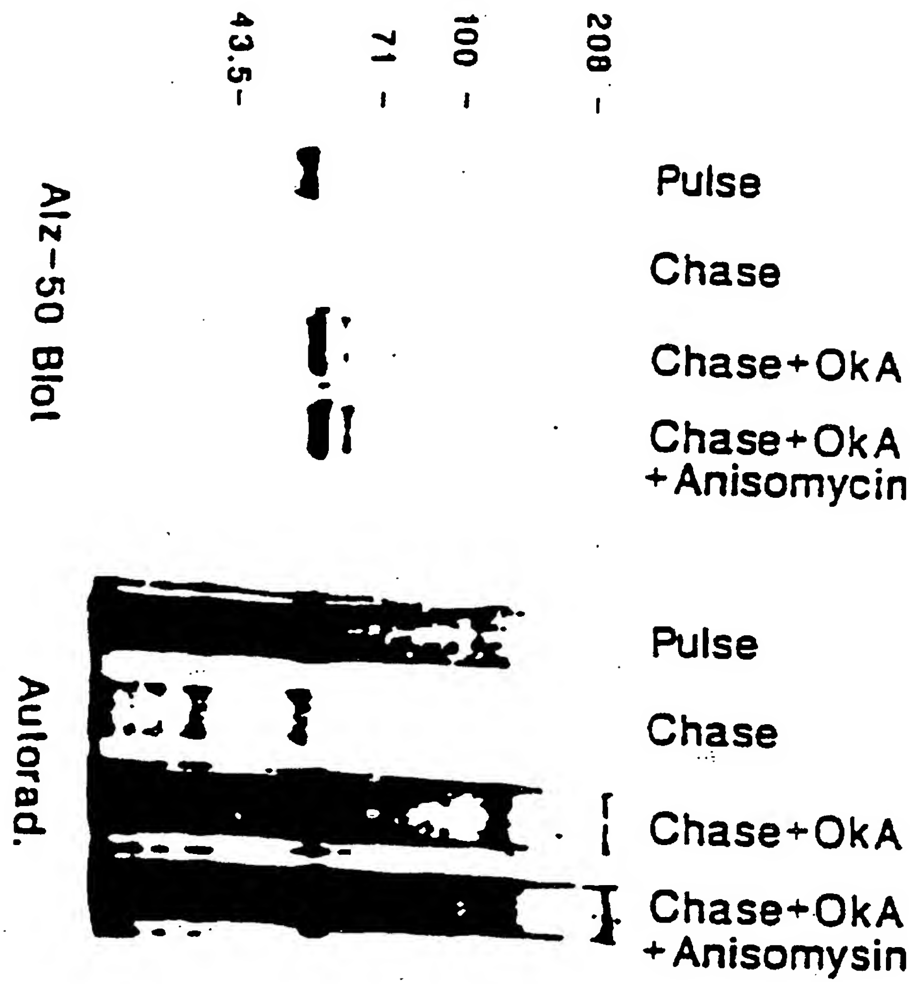

Alkaline phosphatase
-
+
-
+
Control 0min
Control 60min
10μM OkA 60min
Control 0min
Control 60min
10μM OkA 60min
Control 0min
Control 60min
50 nM OkA 60min
10μM OkA 60min
Control 0min
Control 60min
50 nM OkA 60min
10μM OkA 60min
Alz-50
Tau-1
200-
117-
76-
48-
188
PHF-1
T3P
NP-8

FIGURE 4


Control
1μM OkA
1μM OkA + 100uM Anisomycin
Control
1μM OkA
1μM OkA + 100uM Anisomycin
Control
1μM OkA
1μM OkA + 100uM Anisomycin
Alz-50
Tau-1
188
200-
117-
76-
48-
PHF-1
NP-8
T3P
200-
117-
76-
48-

FIGURES 5A and 5B




43.5-
71 -
100 -
208 -
Alz-50 Blot
Pulse
Chase
Chase+OkA
Chase+OkA
+Anisomycin
Autorad.
Pulse
Chase
Chase+OkA
Chase+OkA
+Anisomysin

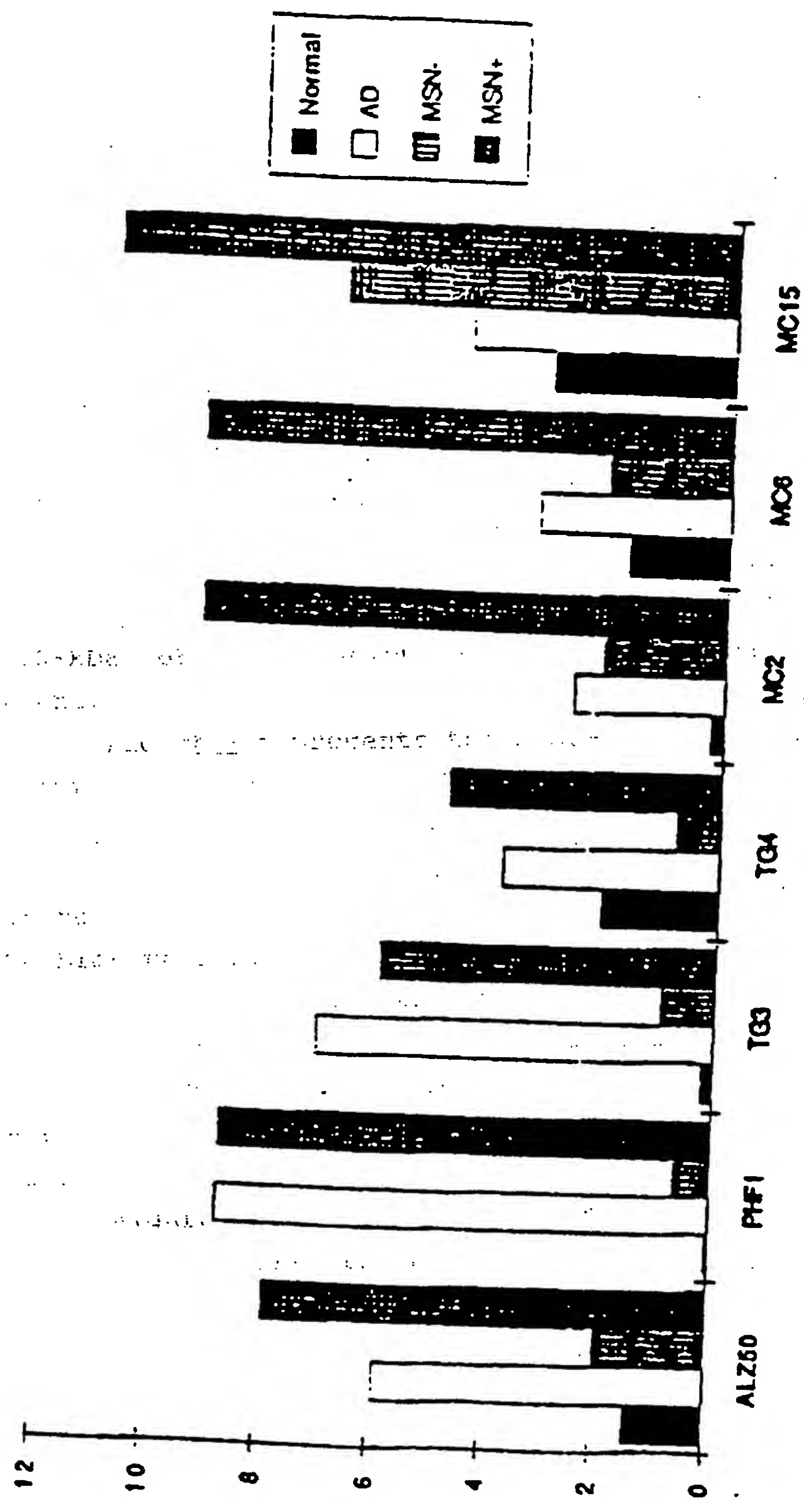


FIGURE 7

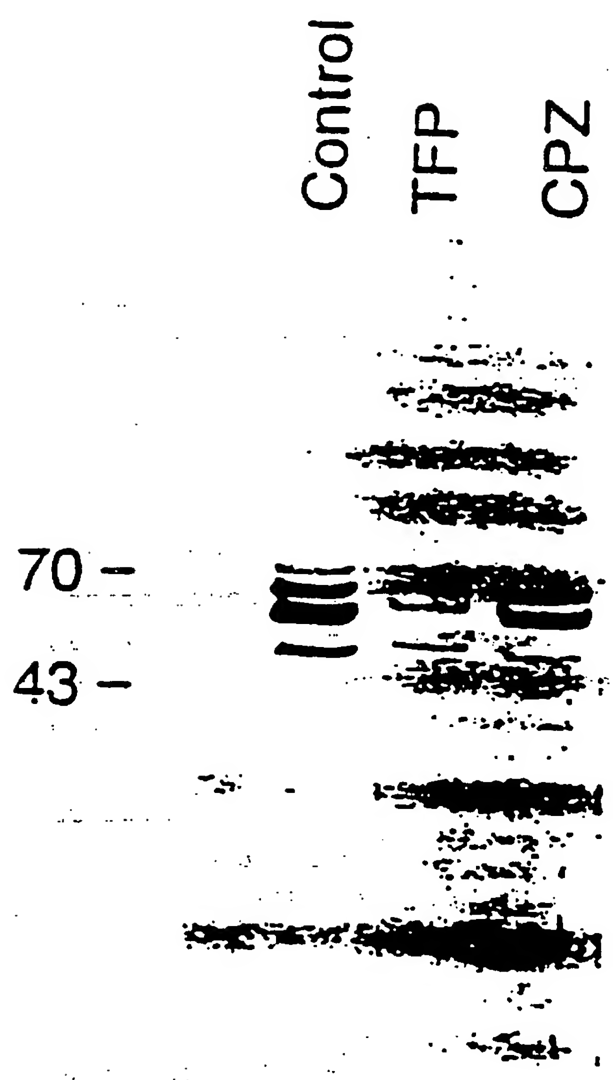

Control
TFP
CPZ
70 –
43 –

FIGURE 9A

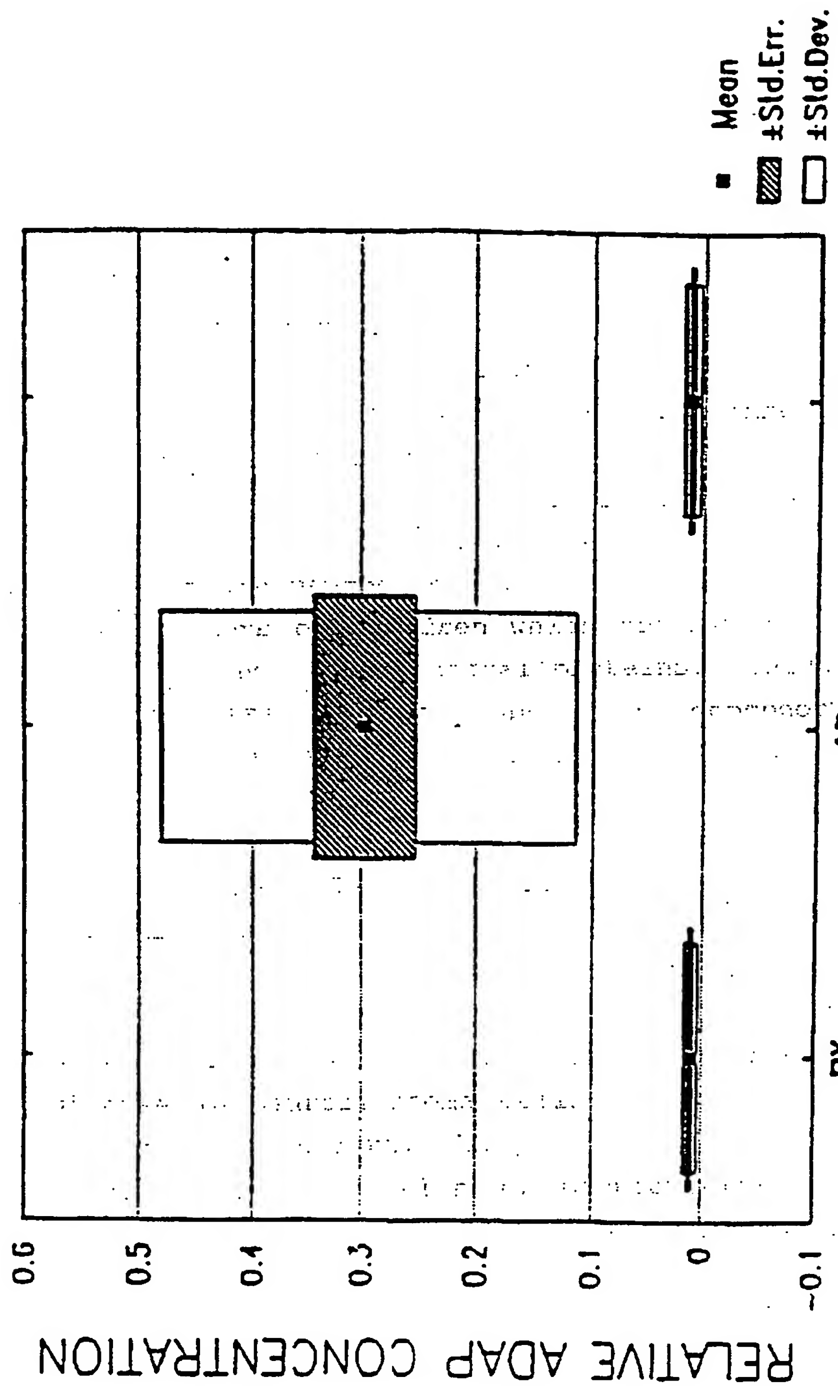
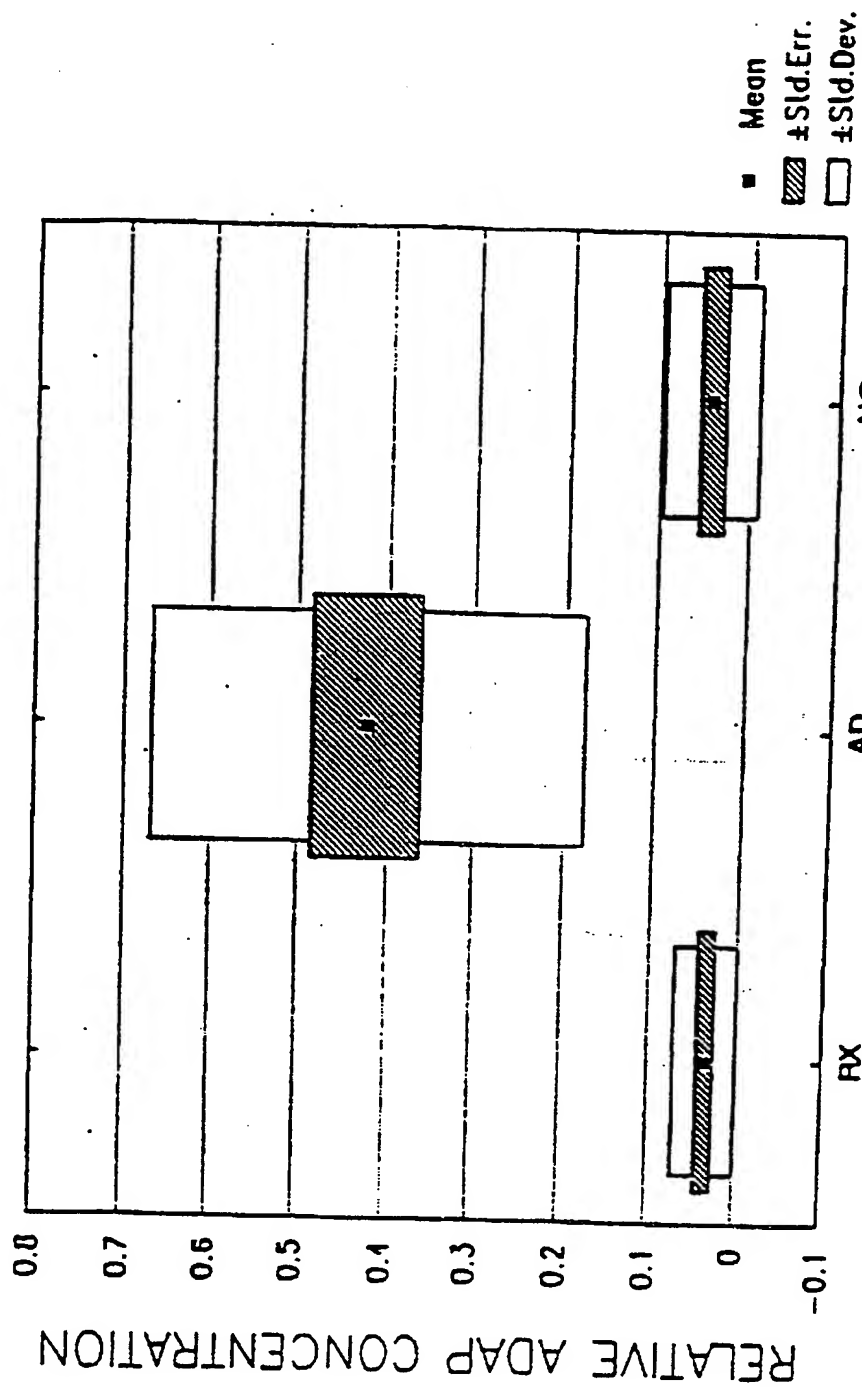

RELATIVE ADAP CONCENTRATION
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
RX
AD
NC
Mean
±Std.Err.
±Std.Dev.

FIGURE 9B


RELATIVE ADAP CONCENTRATION
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
RX
AD
NC
Mean
±Std.Err.
±Std.Dev.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/10110

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/54 A61K31/38 A61K31/495

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EXP. NEUROL. , vol. 125, no. 2, 1994 pages 286-295, A.J. ANDERSON ET AL. 'Increased immunoreactivity for Jun- and Fos-related proteins in alzheimer's disease: association with pathology.' ---	
A	ON CONTINUING PRACTICE, vol. 12, no. 2, 1985 pages 11-13, C. RYMES-BARLEY ET AL. 'Alzheimer's disease- are drugs really effective?' ---	

-/--

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search
22 November 1995

Date of mailing of the international search report
12. 12. 95

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Authorized officer
Klaver, T

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 95/10110

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AM. DRUG., vol. 202, no. 3, 1990 pages 78-88, W.G. ERWIN ET AL. 'Treatment of Alzheimer's disease.' -----	

1

INTERNATIONAL SEARCH REPORT	International application No. PCT/US95/10110

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: "Remark: Although
because they relate to subject matter not required to be searched by this Authority, namely:
all claims are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition."

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.